University of Alberta

PROMOTING PLASTICITY IN THE INJURED SPINAL CORD

by

Jacklyn Girgis

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ABSTRACT

Spinal cord injury (SCI) is a devastating event resulting in the loss of motor and sensory function below the level of the lesion. Regeneration of injured axons is limited in the central nervous system (CNS) due to several factors including the inhibitory environment of the injured white matter. Cortical and/or spinal adaptive changes may occur spontaneously following partial injury. My goal was to elucidate methods of promoting functional recovery following SCI and to determine the mechanism(s) for the observed recovery. In the first project, rats were given neurotrophins to enhance the formation of new connections within the injured spinal cord. In the second project, specific (grasp) training was given to animals with cervical SCI in order to promote functional recovery. In both projects, treatments resulted in spinal and/or cortical adaptive changes which led to functional improvements.

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TABLE OF CONTENTS

Chapter 1 – Introduction 1-35

1.1 Spinal cord injury 2

1.2 Spontaneous recovery following spinal cord injury 3

1.3 Factors preventing regeneration 7

1.4 Treatments 11

1.4.1 Neurotrophins 11

1.4.2 Exercise 15

1.5 Concluding remarks 21

1.6 Figures 23

1.7 Reference list 27

Chapter 2 – BDNF promotes connections of corticospinal neurons onto spared descending interneurons in spinal cord injured rats 36-75

2.1 Introduction 37

2.2 Materials and methods 39

2.2.1Animals 39

2.2.2 Lesions 40

2.2.3 Neurotrophin application 41

2.2.4 Behavioural testing 42

2.2.5 Tracing 43

2.2.6 Anatomical analysis 44

2.2.7 Statistics 48

2.3 Results 48

2.3.1 Most propriospinal interneurons linking the cervical and lumbar enlargements are commissural 48

2.3.2. Only BDNF application together with injury increases collateral sprouting at the cervical level 50

2.3.3 Bouton-like structions of CST collaterals contact propriospinal interneurons 52

2.3.4 BDNF induced collaterals are not specifically targeting PrI 53

2.3.5 Neither injury alone nor the addition of BDNF increase sprouting of injured CST fibers at thoracic level 54

2.3.6 Increase in CST-PrI contacts correlates with functional recovery .. 55

2.4 Discussion 55

2.5 Figures 60

2.6 Reference list 71

Chapter 3 – Reaching training promotes plasticity in parallel with functional recovery following cervical spinal cord injury in rats 76-127

3.1 Introduction 77

3.2 Materials and methods 79

3.2.1 Animals 79

3.2.2 Single pellet reaching task 80

3.2.3 Qualitative analysis of skilled reaching 81

3.2.4 Horizontal ladder walking task 82

3.2.5 Spinal cord injury 82

3.2.6 Post-injury training 83

3.2.7 Final testing 84

3.2.8 Electrophysiology 85

3.2.9 Tracing 87

3.2.10 Perfusions and histology 87

3.2.11 Statistics 89

3.3 Results 90

3.3.1 Lesion size between trained and untrained rats is comparable 90

3.3.2 Training increases single pellet reaching task success rate in CST/RST injured animals 91

3.3.3 Training does not alter grasping style in CST/RST injured rats 92

3.3.4 Increased occurrence of motor evoked potentials in trained CST/RST injured rats 93

3.3.5 Training does not alter thresholds or delays in injured rats 94

3.3.6 Training increases the number of CST collaterals in cervical enlargement of CST/RST injured rats 95

3.3.7 Reaching training interferes with ladder walking 96

3.4 Discussion 96

3.4.1 CST/RST injured rats 97

3.4.2 RST injured rats 103

3.4.3 CST injured rats 106

3.4.4 Contribution of the CST and RST in the single pellet reaching task 107

3.4.5 Concluding remarks 108

3.5 Figures 109

3.6 Reference list 124

Chapter 4 – Discussion 128-141

4.1 Major findings 129

4.2 Application of the results 132

4.3 Future directions 134

4.3.1 Chapter 2 future directions 134

4.3.2 Chapter 3 future directions 135

4.4 Concluding remarks 137

4.5 Figures 139

4.6 Reference list 141

LIST OF TABLES

Table 2.1: Averages of collateral counts at the cervical level 70

LIST OF FIGURES

Figure 1.1: Schematic of the inhibitory environment of the CNS 23

Figure 1.2: Schematic of the study from Chapter 2 25

Figure 1.3: Schematic of the study from Chapter 3 26

Figure 2.1: Location of PrI within the cervical enlargement following thoracic spinal cord injury 60

Figure 2.2: Distribution of PrI in the cervical and thoracic spinal cord 61

Figure 2.3: Corticospinal tract collaterals at the cervical level in BDNF and NT-3 treated animals as well as controls 62

Figure 2.4: CGRP fiber density in NT-3 and control animals 63

Figure 2.5: PrI contacts in the cervical enlargement 64

Figure 2.6: Projection of corticospinal tract projections in the cervical enlargement 66

Figure 2.7: Corticospinal tract collaterals and contacts onto PrI in the thoracic spinal cord 68

Figure 2.8: Correlation between number of contacts and error rate on the horizontal ladder task 69

Figure 3.1: Schematic of the single pellet reaching task chamber 109

Figure 3.2: Timeline for the study 110

Figure 3.3: Lesion size analysis 111

Figure 3.4: Single pellet reaching task success rate 112

Figure 3.5: Detailed grasping style analysis in CST/RST and RST only injured animals 113

Figure 3.6: Cortical map (contralateral to injury) in CST/RST and RST only injured animals 115

Figure 3.7: Cortical map (ipsilateral to injury) in CST/RST and RST only injured animals 117

Figure 3.8: Minimum threshold to elicit a motor evoked potential in the injured forelimb following stimulation of the contralateral cortex 119

Figure 3.9: Sample EMG trace and delay to elicit a forelimb movement in CST/RST and RST only injured animals 120

Figure 3.10: Number of CST collaterals emerging from the injured CST rostral to the injury 121

Figure 3.11: Error rates on the horizontal ladder walking task 123

Figure 4.1: Schematic of the study from Chapter 2 139

Figure 4.2: Schematic of the study from Chapter 3 140

List of Abbreviations

ABC – Avidin biotin complex

BBB score – Basso, Beattie & Bresnahan behavioural score for contusive spinal cord injury

BDA – Biotinylated dextran amine

BDNF – Brain derived neurotrophic factor

C - Cervical

Ca^{2+} – Calcium

cAMP – Cyclic adenosine monophosphate

CGRP – Calcitonin gene-related peptide

CIMT – Constraint induced movement therapy

CNS – Central nervous system

CPG – Central pattern generator / Central pattern generating network

CST – Corticospinal tract

DAB - 3,3-diaminobenzidine

DRG – Dorsal root ganglia

FGF – Fibroblast growth factor

FR – FluoroRuby

GAP-43 – Growth associated factor 43

GDNF – Glial cell-line derived neurotrophic factor

L - Lumbar

MAG – Myelin associated glycoprotein

MEP – Motor evoked potential

NgR – Nogo receptor

NT-3 – Neurotrophic factor 3

OMgp – Oligodendrocyte myelin glycoprotein

PKC – Protein kinase C

PNS – Peripheral nervous system

PrI – Propriospinal interneurons

RtST – Reticulospinal tract

RST – Rubrospinal tract

SCI – Spinal cord injury

TBS – Tris-buffered saline

Th – Thoracic

CHAPTER 1

INTRODUCTION: PROMOTING PLASTICITY IN THE INJURED SPINAL CORD

1.1 Introduction

Spinal cord injury (SCI) is a devastating event which results in the loss of motor and sensory function below the level of the lesion. In the United States of America alone, there are approximately 300,000 people living with SCI with 12,000 new injuries occurring each year. The leading cause of SCI is accidents (motor vehicle, sports etc), and the average age of a person affected with SCI is 33.4 years old with most injuries occurring at the age of 19 (National Spinal Cord Injury Statistical Center (NSCISC)). People living with SCI only have an average reduction in life expectancy of 10%, meaning they could be living wheelchair-bound for 40 or more years. The impact on the quality of life of a person suffering from a SCI is immeasurable, but there is a large economic burden as well. A SCI patient in the United States of America who sustains an injury at 25 years of age may end up with up to $3 million in healthcare costs throughout their life (NSCISC).

Since the 1980's there has been a surge in the knowledge of the events following SCI. Previously, it was unknown whether the central nervous system (CNS) was capable of repair (regeneration and/or sprouting) following injury. It has been established that following injury to the peripheral nervous system (PNS) regeneration of injured axons occurs readily. Several seminal papers in the 1980's and 1990's have shown that it is likely the inhibitory environment of the injured CNS (and not the CNS neurons themselves) that limits regeneration following injury (David & Aguayo, 1981; Schwab & Thoenen, 1985; Schwab & Caroni, 1988). Following PNS injury, macrophages and Schwann cells work quickly to remove debris (forming the majority of the inhibitory environment) caused by the injury and Schwann cells work to guide axons back to their

original targets. This occurs quite slowly or not at all following injury to the CNS (as reviewed in Fenrich & Gordon, 2004).

Another important factor preventing regeneration in the CNS is growth inhibitory molecules in myelinating cells of the CNS (oligodendrocytes) and also in damaged myelin (see section 1.3). Although some of these growth inhibitory molecules are found within the PNS (e.g. MAG on Schwann cells), many more are found within the CNS. Also, as explained earlier, Schwann cells and macrophages work quickly to remove myelin debris within the PNS, whereas removal of myelin occurs much slower within the injured CNS. Furthermore, following CNS injury (but not PNS injury), a glial scar is formed (described in section 1.3). Even with all of these factors influencing regeneration following SCI, some spontaneous recovery of function may occur following partial injury.

In this chapter, I will describe this spontaneous recovery of function, as well as some factors known to limit recovery which have been mentioned above. I will also discuss approaches to promote functional recovery by repairing the injured spinal cord. Chapters 2 and 3 will describe two separate projects completed during my Master's thesis work which were aimed at promoting functional recovery following SCI. In chapter 4, the implications of the results from chapters 2 and 3 will be discussed in detail.

1.2 Spontaneous Recovery Following Spinal Cord Injury

Following incomplete SCI, some recovery of function may occur spontaneously. Mechanisms of short term recovery include regaining the ionic balance in the spinal cord (a factor important in promoting the cessation of spinal shock), and the reappearance of

motor neuron excitability. Immediately following trauma to the spinal cord, the ionic balance surrounding axons is severely altered due to disruption of the blood brain barrier and also damage to myelin. Demyelination occurs following SCI due to both the initial trauma to the spinal cord (severing axons and myelin) and also the damage done to oligodendrocytes, preventing remyelination of the axons (Yiu & He, 2006). The shift in ionic balance, as well as the demyelination found after injury may prevent the propagation of action potentials (Hulsebosch, 2002). Furthermore, following demyelination caused by SCI, the distribution of ion channels is altered causing severe disturbances in axonal function. Remyelination, however, can restore the pattern of ion channel distribution to within nodes of Ranvier, promoting some return of axonal function following injury (Black et al., 2006). Remyelination of spared axons has also been shown to promote functional recovery following SCI. Jeffrey and Blakemore (1997) showed that rats subjected to a demylination injury could fully recover behaviourally as long as remyelination occurred.

Spinal shock (which is due, in part to ionic shifts found after SCI), occurs acutely (within hours to days depending on species and injury severity) and is defined as flaccid paralysis and loss of tendon reflexes below the level of the lesion. A decrease in motor neuron excitability is most likely involved in the formation of spinal shock (Hiersemenzel et al., 2000). Following SCI, motor neurons lose their supraspinal inputs and subsequently no longer receive neuromodulators such as serotonin and norepinephrine that they require in order to function (Bennett et al 2004).

There are several proposed mechanisms of long-term recovery (weeks to years after injury) following SCI. It has been well-documented that compensatory movement

strategies are acquired in the months and years following partial SCI (McKenna & Whishaw, 1999). It is thought that perhaps anatomical changes within the injured spinal cord may be related to this recovery. Axonal sprouting, the growth or sprouting of collaterals emanating from an axon and entering the grey matter, is one of these proposed anatomical changes within the spinal cord. Axonal sprouting has been found to occur spontaneously following partial thoracic lesions in the spinal cord. Sprouting has been found both rostral (Fouad et al., 2001) and caudal (Ballermann et al., 2006; Weidner et al., 2001) to a spinal lesion. A recent report has also shown that the spinal cord is capable of making meaningful anatomical rearrangements spontaneously following partial injury. Bareyre et al (2005) showed that following a partial dorsal lesion in the thoracic spinal cord, the corticospinal tract (CST) increased the number of collaterals entering into the grey matter in the cervical enlargement. Those collaterals increased their connections onto long descending propriospinal interneurons (terminating in the lumbar enlargement) at the same time, leading to new intra-spinal connections. Ablation of the new connections (relesion of the CST) was shown to eliminate the spontaneous functional recovery seen in the animals, suggesting that the connections promoted the recovery. Relesions (especially following partial injury) are impossible to verify (i.e. they may cause more injury than was done with the first injury or they may not ablate new connections made after the first injury), and so it is unclear whether the spontaneous recovery found after SCI was due to the formation of new connections within the spinal cord. In Chapter 2, a later study is described in which the number of collateral-interneuron connections was found to correlate with behavioural improvements found after SCI. The connections in this study were not formed spontaneously, however, but

were promoted by the addition of a neurotrophic factor. Nevertheless, it appears that the formation of new connections within the spinal cord is sufficient to promote recovery of function.

Other mechanisms related to the formation of compensatory movement strategies include disinhibition in pattern generating networks (de Leon et al., 1999), the exploitation of previously unused synaptic pathways (de Leon et al, 2001) and changes in the cortical representation for a specific limb (i.e. changes cortical maps; Bruehlmeier et al., 1999). The term plasticity is often used as an umbrella term incorporating all the previously mentioned adaptive changes.

Changes in the cortical representation (i.e. cortical maps) for a specific limb in the sensory-motor area of the cortex are another mechanism promoting plasticity. Cortical map changes have been found following brain injury (Jones and Schallert, 1994; Bury and Jones, 2002), peripheral nerve injury (Wu and Kaas, 1999) and SCI (Bruehlmeier et al., 1998; Fouad et al., 2001; Turner et al., 2003), in humans and animal models. Commonly following injury, the size of the cortical representation corresponding to the limb with the injury will decrease, while the size of the cortical representation of the other limb will increase. The reason for this is two-fold. First, a decrease in the size of the cortical map for the injured limb is likely due to disuse of the limb, and also a decrease in sensory information returning to the brain from the periphery. Second, an increase in the cortical map for the other (uninjured) limb may be related to an increase in the use of this limb and also an increase in sensory information returning to the cortex. This can be evidenced by examining cortical maps following the forced use of an injured limb (for example, by using constraint induced movement therapy; CIMT; see section 1.4.2). In

humans, when use of an injured limb is promoted with CIMT, reductions in the size of the cortical maps corresponding to that limb are eliminated (Ro et al., 2006).

Although spontaneous injury-induced adaptive changes occur following a SCI (as detailed above), injured axons attempting to regenerate in the CNS still face many varied obstacles which prevent recovery. These factors detailed below include inhibitory molecules within the injured spinal cord and their downstream effectors.

1.3 Factors Preventing Regeneration Following Spinal Cord Injury

Until recently, it was unknown why CNS neurons did not regenerate following injury. Researchers theorized that it was either an intrinsic inability of CNS axons to regenerate or that the environment of the injured CNS was inhibitory to growth. Several *in vitro* studies in the late 1970s and early 1980s provided evidence that supported the idea of the lack of regenerative ability being due to the environment rather than the axons. This idea, which was originally suggested by Cajal (1928), was supported by the findings that axons from the PNS (with a known regenerative ability) were unable to regenerate onto CNS glia (Schwab & Caroni,1988) and that CNS axons were able to regenerate when placed into peripheral nerve grafts (David & Aguayo, 1981) but will not grow through/onto CNS grafts (Schwab & Thoenen, 1985). It has been shown that not all of the CNS is inhibitory to growth, and that the CNS white matter (but not the grey matter) is the cause of the inhibitory environment (Savio & Schwab 1989). While the inhibitory environment of the CNS was being elucidated, antibodies towards yet uncharacterized components of myelin were made (IN-1 and IN-2) and were shown to reduce the hostile environment of the CNS white matter (Caroni & Schwab 1988). It was not until over 10 years later that Nogo

was discovered and found to be an antigen to IN-1 (Chen et al., 2000). Two other major myelin based inhibitors (myelin associated glycoprotein; MAG (McKerracher et al., 1994) and oligodendrocyte myelin glycoprotein; OMgp (Kottis et al., 2002)) have also been discovered. Much to the surprise of researchers in the field, all three of the myelin associated growth inhibitors acted through the same receptor, the Nogo receptor (NgR; named after its first discovered function; Domeniconi et al., 2002; Fournier et al., 2001; Wang et al., 2002).

The growth permissive effects of inhibiting the myelin associated growth inhibitors has been shown both *in vitro* and *in vivo* with varying degrees of success. Another approach to demonstrate the role of a myelin associated inhibitor is to create an animal lacking an inhibitor. Three separate groups have created transgenic mice lacking Nogo. In all cases the animals were able to survive to adulthood. The results were very different between the groups, ranging from no effect to a strong positive effect on axonal regeneration *in vivo* (Zheng et al., 2003; Simonen et al., 2003; Kim et al., 2003). The reason(s) for the different outcomes are still unclear (Woolf 2003) but may include differences in the methods of inducing the knockouts.

Although a lot of attention has been placed on the growth inhibitory effects of Nogo, OMgp, MAG and their receptor NgR it is now known that the pathway for the myelin inhibitors is far more complex than originally thought. Specifically, several co-receptors act with NgR (p75, TROY, LINGO 1) and several other molecules (with their own receptors) have been found to be inhibitory to CNS regeneration (e.g. ephrin, semaphorin 4D, semaphorin 3A as well as the epidermal growth factor receptor; as reviewed in Yiu & He, 2006).

As shown in Figure 1.1, the binding of ligands to NgR causes the activation of several downstream signals including increased intracellular Ca^{2+} as well as RhoA (Yiu & He, 2006). RhoA is one of a family of many small GTP-ases whose main action is on actin rearrangement. Although the exact mechanism of axonal growth has not been fully elucidated, it is well known that actin rearrangements are required for both axonal growth and for the growth cone leading the axon to progress (i.e. move and turn) in the environment (Raftopoulou & Hall 2004). Once in its active (GTP-bound) state, RhoA acts Rho-associated kinase (ROCK) to stimulate growth cone collapse and subsequently blocks axon elongation. Inactivation of Rho is sufficient to promote neurite outgrowth on inhibitory substrates *in vitro* (Lehmann et al., 1999; Dergham et al., 2002; Fournier et al., 2003) and axonal regeneration *in vivo* following optic nerve crush (Lehmann et al., 1999) and SCI in mice (Dergham et al., 2002). Inhibition of ROCK is also sufficient to promote neurite extention on inhibitory substrates *in vitro* (Dergham et al., 2002; Fournier et al., 2003) and promotes regeneration following SCI in mice (Dergham et al., 2002) and axonal sprouting following SCI in rats (Fournier et al., 2003).

Results of functional recovery following SCI in both mice and rats treated with Rho pathway inhibitors are more complex. In mice, inhibition of either Rho or ROCK promoted both regeneration of injured CST axons and recovery of walking ability (Dergham et al., 2002). In rats, however, only ROCK inhibition promoted sprouting of CST fibers and only accelerated recovery of walking ability compared to untreated rats (Fournier et al., 2003). Concentrations and delivery of the inhibitors differed substantially between the two studies, which may account for some of the variability. Furthermore, the behavioural task used (the Basso, Beattie & Bresnahan score; BBB score; Basso et al.,

1995) was applied differently in both studies. Specifically, in rats, the full 21-point score was used, whereas a modified version of the score was used in mice due to an inability to accurately score certain components of walking behaviour in mice (due to their smaller size). Nevertheless, all 3 of the studies mentioned above do show that blockade of the Rho pathway can provide benefit (regeneration, sprouting and functional recovery) following experimental CNS injury (SCI or optic nerve crush).

Another important factor preventing regeneration following SCI is the scar formed around the injury. Depending on the integrity of the dura matter, the glial scar can have two different compositions. In the case of an intact dura matter (i.e. compressive or contusive injuries), the scar is made up of primarily of astrocytes, but when the dura matter is compromised (i.e. following cut injuries), the astrocytes then become mixed with invading elements such as connective tissue (Silver & Miller, 2004). The response of astrocytes to injury is called reactive gliosis, and is characterized by the strengthening and growth of the astrocytes. Following injury, reactive astrocytes produce several classes of proteoglycans, of which chondroitin sulfate proteoglycans (CSPGs) have been studied extensively (Laabs et al., 2005). CSPGs are not only produced in response to injury, but exist in the healthy maturing CNS as an inhibitory barrier for extending axons (e.g. in the optic chiasm and the dorsal root entry zone; Laabs et al., 2005). Their presence in the injured CNS prevents the growth of axons in or around the scar (Silver & Miller, 2004). When axons come in contact with the glial scar, dystrophic endbulbs form, causing collapse. One of the CSPGs (versican V2) has been shown to act by increasing RhoA via a p75/NgR independent mechanism (Schweigreiter et al., 2004), but a receptor specifically for CSPGs has not been found. Nevertheless, attempts to digest the

glial scar itself (by use of the enzyme chondroitinase) have been promising in promoting regeneration of injured axons and functional recovery (Bradbury et al., 2002; Fouad et al., 2005).

1.4 Treatments Aimed At Promoting Functional Recovery Following Spinal Cord Injury

Due to the various factors preventing recovery detailed above, it has been difficult to find therapeutic treatments for SCI. Methylprednisolone (meant to reduce the immune response following SCI) is the most commonly administered treatment following human SCI (Bracken et al., 1990), but its use remains controversial due to inconclusive results in drug trials where certain patients acquiring myopathies (Qian et al., 2005). This type of treatment, however, is not in keeping with the topic of the current thesis and will not be discussed any further. Detailed below are two treatments currently being explored as possible therapeutic treatments following human SCI: neurotrophins and exercise. The two treatments were examined for my Master's thesis and make up the two projects completed during my degree.

1.4.1 Neurotrophins

Neurotrophins are a group of proteins that are essential for the growth and development of the vertebrate nervous system (Chao, 2003). The neurotrophins act through three main neurotrophin receptors (TrkA for nerve growth factor (NGF), TrkB for NT-4 and brain-derived neurotrophic factor (BDNF) and TrkC for NT-3) as well as a fourth receptor that has equal affinity for all neurotrophins (p75 receptor).

In terms of their effects on regeneration and/or sprouting following injury, neurotrophins have been linked via affecting the response of the growth cone to myelin based inhibitors. Neurotrophic factors (BDNF, glial cell-line derived neurotrophic factor (GDNF) and NGF) have been shown to reduce inhibition of MAG *in vitro* (Cai et al.,1999). This effect was found to be dependent upon cyclic adenosine monophosphate (cAMP), in that blocking one of the downstream effectors of cAMP reversed the beneficial effects of the neurotrophins. In a previous study, cAMP was shown to modulate the effect of a guidance cue (netrin-1). Specifically, netrin-1 became chemorepulsive without adequate cAMP present (Ming et al., 1997). The combination of cAMP (injected into the dorsal root ganglia (DRG)) and NT-3 (injected directly into the spinal cord) promoted the growth of sensory fibers into the spinal cord and beyond the lesion site (Lu et al., 2004). Furthermore, cAMP has been shown to be sufficient to reduce functional deficits following experimental SCI (Nikulina et al., 2004). The effects of another guidance cue, semaphorin 3A (repulsive guidance cue), are modulated in part by neurotrophins, or specifically their receptors. Activation of TrkA (with NGF) appears to decrease the sensitivity of growth cones to the inhibitory semaphorin 3A whereas TrkB activation (with BDNF) has the opposite effect (Tuttle & O'Leary, 1998). Therefore, it appears that neurotrophins can alter neuronal response to the environment partly by a cAMP-dependent mechanism.

Neurotrophins have been applied *in vivo* after SCI and PNS injury. NT-3 has been shown to both increase (Schnell et al., 1994; Zhou et al., 2003) and decrease (Hagg et al., 2003) local sprouting of the corticospinal tract. It appears that the location of NT-3 application is particularly important in terms of the effects it will have on the spinal cord

after injury (detailed in chapter 4). The application of cells engineered to express NT-3 (via a viral vector) has been shown to reduce degeneration following SCI and to increase both the sparing and regeneration of the CST (Ruitenberg et al., 2005). Furthermore, grafting of a peripheral nerve modified to express NT-3 (Blits et al., 2000) or a grafted bridge composed of fibroblasts modified to express NT-3 (Grill et al., 1997) was sufficient to promote regeneration of the CST and functional recovery following SCI.

Application of BDNF to the cell body of injured tracts has been shown to increase factors important to cell growth (e.g. growth associated protein 43; GAP-43) and to promote sprouting of injured axons (Kobayashi et al., 2003). Furthermore, BDNF applied to the cell body has been shown to increase sprouting of the CST (Hiebert et al., 2002). In Chapter 2, I will describe how BDNF application to the cell body was also found to increase connections of these sprouts onto propriospinal interneurons.

To provide the most benefit following SCI, it appears that combining several neurotrophins may be best. In a recent study, only when three neurotrophins (fibroblast growth factor (FGF), BDNF and NT-3) were given in concert to the retinal cell body did regeneration proceed into the CNS (Logan et al., 2006). Previously, it has also been shown that combining two neurotrophins (BDNF and GDNF) provides benefit (PNS regeneration) over and above the sum of both of the neurotrophins on their own (Boyd & Gordon, 2003). Furthermore, BDNF appears to have a dose-dependent action following PNS injury in that at lower doses it promotes regeneration but at higher doses it actually impedes recovery. As reported with the effects of neurotrophins on semaphorin 3A repulsion (Tuttle & O'Leary, 1998), the effect of BDNF on PNS regeneration appears to be differentially regulated by the two classes of neurotrophin receptors. Blockade of the

p75 receptor reversed the negative effects of high dose BDNF treatment (Boyd & Gordon, 2002). Furthermore, heterozygous TrkB knockout mice had significantly reduced PNS regeneration compared to wild-type mice (Boyd & Gordon, 2001).

The evidence reported above shows that neurotrophic factors can be used to enhance regeneration or sprouting following SCI or PNS injury and furthermore, may promote functional recovery following SCI. Building upon the literature showing axonal sprouting, regeneration and functional recovery following application of neurotrophins, the study in Chapter 2 (see Figure 1.2) was conducted to determine whether neurotrophins could enhance the formation of intraspinal connections found spontaneously following SCI (Bareyre et al., 2004). Furthermore, we wanted to determine whether specific/targeted application of a neurotrophin could provide benefit (i.e. BDNF application directly to the cell bodies of CST axons) as this would circumvent possible side effects.

Finding ways of promoting regeneration/sprouting onto distinct descending tracts is of great importance, especially following human SCI. Pain is a common correlate of SCI, and a global treatment promoting sprouting may increase sprouting of ascending sensory fibers, leading to increased sensation of pain (Ro & Chang, 2005). Due to this, and the many other factors influencing benefit and detriment following neurotrophic factor treatment, it has not yet been accepted as a treatment following human SCI or PNS injury. A treatment which is already in use following cases of human SCI is exercise. In the next section, I will describe how and why exercise has been used following experimental SCI and other CNS diseases, and how it has been adapted to the clinical situation.

1.4.2 Exercise

Physical activity has been used as a treatment following several different types of injuries, including stroke and SCI. In animal models, there are many ways of increasing physical activity including constraint induced movement therapy (CIMT), treadmill training, running wheel training and environmental enrichment. Following increased physical activity BDNF, NT-3 protein as well as mRNA and NT-4 protein have been found to be increased in the spinal cord (Gomez-Pinilla et al., 2001, 2002; Skup et al., 2002; Ying et al., 2003, 2005). Importantly, there are also increases in two neurotrophin receptors (TrkB and TrkC) in the spinal cord following running training (Gomez-Pinilla et al., 2001; Skup et al., 2000; Ying et al., 2003). GAP-43 (a growth-associated protein which has been found in the growth cone of regenerating axons (Azcurra et al., 2003)) has also been found to be upregulated in the brains of animals following training. Furthermore, the Nogo receptor (NgR) is downregulated following increased activity (Josephson et al., 2003). Therefore, increased physical activity appears to increase factors implicated in promoting plasticity (e.g. GAP-43, and neurotrophins) while decreasing a factor implicated in preventing regeneration and sprouting (i.e. NgR).

Treadmill training is used frequently in animals and humans to increase physical activity following SCI. Treadmill training has its foundation rooted in research on cats with completely transected spinal cords (spinalized cats). Spinalized cats can re-learn to step and support their weight on a treadmill following training (Lovely et al., 1986). Although the mechanisms of recovery are not fully known, it is likely due to disinhibition of pattern generating networks (de Leon et al., 1999) and plasticity (sprouting above and below the lesion) which may be related to the formation of compensatory movement

strategies (see above; Mckenna & Whishaw, 1999). Importantly, the recovery found following training in cats appears to be task specific. For example, spinalized cats trained to stand cannot walk on a treadmill as fast as spinalized cats trained to step (Edgerton et al., 1997; de Leon et al., 1999). In humans, this same pattern appears to be evident. Specifically, SCI patients trained to walk forwards on a treadmill with body weight support regained a steady walking pattern in terms of muscle activation and kinematic analysis (compared to controls). When the direction of the treadmill belt was reversed, and patients were asked to walk backwards, they were unable to do so. Similarly, patients were also unable to perform stepping motions on a stationary treadmill (Grasso et al., 2004). It is important to note that hindlimb stepping is orchestrated via a pattern generating network (CPG). Accordingly, training forward walking, or standing would effectively alter this network and may explain why only trained actions are improved following SCI.

CIMT, another mechanism to increase physical activity, is based on the premise of "use it or lose it", where locomotor abilities and even neuronal connections are thought to be lost when they are no longer used (Shallert et al., 2000). This was originally found following a primate model of limb deafferentation, wherein the animal relies primarily on the intact limb and avoids use of the other limb (reviewed in Taub, 1999). There are two main ways of combating this effect by use of CIMT. One approach is the restraining of the uninjured limb, forcing the animal to use the injured limb, with the belief that the use of the limb will increase the performance of daily activities. Another line of research focuses not only on the restraining of the uninjured limb concurrent with the performance of specific (usually grasping) tasks with the injured limb. It is not clear which method

provides more benefit following injury, but each method has its drawbacks. For example, by only restraining the uninjured limb, there may not be sufficient physical activity in the injured limb to promote recovery. On the other hand (as detailed below) excessive use of the injured limb after a recent injury can be detrimental.

Although both CIMT and treadmill training have been reported to promote functional recovery in animal (DeBow et al., 2003; Lovely et al., 1986) and human studies (Beekhuizen et al., 2005; Harkema, 2005), it appears that multiple factors come into play in determining the amount of success (or even detriment) the treatments will have. In the case of CIMT, one highly important factor appears to be the delay between injury and implementation of the treatment (Humm et al., 1998). Specifically, animals subjected to IPSI-CIMT (i.e. restraint of the uninjured forelimb) immediately following devascularization cortical injuries had increased brain temperatures and increased lesion size compared to animals subjected to CONTRA-CIMT (i.e. restraint of the injured forelimb) at the same time (DeBow et al., 2004). In a separate experiment where animals were given two lesions each (one in the occipital lobe and one in the forelimb motor area of the cortex), only the forelimb area lesion was increased with treatment, suggesting that the increased lesion size is due to excessive movement of the forelimb during a particularly sensitive period following injury.

Similarly, not all studies have reported beneficial effects following treadmill training. For example, Fouad et al (2000) found no increased functional improvement over control rats on any task (including: gridwalk, kinematic analysis, footprint analysis, BBB score, narrow beam and more) after 5 weeks of treadmill training. In cats, however, the results are generally positive, yet fairly rigid in that it appears to be task-specific (see above).

One reason which may account for the difference in results between rodents and cats is self-training. Since rodents are so small and their center of gravity is fairly low, movements of the injured limb can occur more easily. In their home cage it is possible to see rats with very severe lesions still making stepping-like movements with their hindlimbs, where this rarely occurs with cats. If all rats can self-train in their home cages, this can lead to an ineffective control group, which can then lead to negative results.

Another method of exercise similar to treadmill training is the running wheel. Since rodents will use a running wheel spontaneously, even after an injury, the use of a wheel instead of a treadmill may reduce stress to the animal. As with the other forms of exercise, results have been ambiguous. Recently, Engesser-Cesar et al (2006) showed that even the surface of the running wheel can strongly affect the results of running on the wheel. Specifically, animals given access to a running wheel with rungs did not show any functional improvement following spinal cord injury, but animals given access to a running wheel with a flat surface (making it more like a treadmill) did show some functional improvement on an open field (BBB) test and a narrow beam test. This result may relate to the fact that a flat running wheel surface is more similar to an open field where the behavioural testing was completed. Additionally, animals may slip off/between rungs during running, which may cause muscular or skeletal damage to the animal, which would negatively influence their BBB score.

Enriching the environment in which the animal lives is another method of increasing physical activity. In this paradigm, animals are often group housed in cages 2-4 times bigger than standard cages. There are often different levels in the cage with food and water that are only accessible via ropes or ramps to ensure that all animals are indeed

increasing their level of activity. As with all other types of increased physical activity, the results of environmental enrichment are mixed. Several laboratories have reported benefit using environmental enrichment following spinal cord injury (Lankhorst et al., 2001), stroke (Ohlsson & Johansson, 1995), traumatic brain injury (Hamm et al., 1998), Huntington's disease (van Dellen et al., 2000), Alzheimer's disease (Lazarov et al., 2005), Parkinson's disease (Faherty et al., 2005) and epilepsy (Young et al., 1999). Several other laboratories have reported no effect or an exacerbation of injury following environmental enrichment (Erschbamer et al., 2006; Farrell et al., 2001; Gobbo et al., 2005; Jankowsky et al., 2003).

There are many reasons for these conflicting results. First of all, there are no standards for what constitutes enriched environment. Although the majority of reports will group house animals in cages with multiple levels, and will try to promote physical activity as much as possible by placing food and water on different levels, this is not always the case. Some researchers will simply group house the animals in a slightly larger cage with a few toys and stimuli which may or may not promote increased activity. In fact, the different aspects of environmental enrichment (e.g. cognitive/sensory stimulation with toys, exercise and varying motor tasks (climbing, walking etc) have been found to have differential effects on cortical plasticity and working memory in intact mice (Lambert et al., 2005). Secondly, the length of time for animals to be in the enriched environment also varies from weeks to months. Thirdly the delay between injury and implementation of the enriched environment also varies, and some researchers actually place the animals in the enriched environment before injury.

The majority of the above-mentioned treatments based on finding in animal models have been attempted in a clinical setting. It can be easily argued that humans already live in an "enriched environment" and that this type of a "treatment" is already being administered to patients automatically following injury. A common type of treatment following human SCI and stroke (if it affects the lower limbs) is treadmill training, even though the findings in animal models have not been entirely beneficial. No study to date has adequately addressed the functional improvements resulting from treadmill training versus spontaneous functional improvements following SCI, so it is unknown to what degree treadmill training is beneficial. One study has looked at the implications of treadmill training versus overground walking training and it appears that the same level of recovery may occur with either intervention (Dobkin et al., 2006) suggesting that any type of walking training delivered following injury may be beneficial.

Although many reports have documented improved recovery of lower limb function following treadmill training, only one report has documented recovery of upper limb function following use of rehabilitation training following SCI (Beekhuizen & Field-Fote, 2005). Due to the various positive effects exercise has been shown to have following CNS injury (improved recovery (Beekhuizen & Field-Fote, 2005), increased neurotrophins (Ying et al., 2005), increased GAP-43 (Azcurra et al., 2003), downregulation of NgR (Josephson et al., 2003)) the study in Chapter 3 was conducted to see if exercise could have beneficial effects following SCI affecting the upper limbs. We also wanted to determine possible mechanisms for this hypothesized recovery by examining the amount of CST sprouting found after SCI, and the cortical representation for the injured forelimb (i.e. cortical map changes).

1.5 Concluding Remarks

Various anatomical and physiological changes in the CNS following SCI present several obstacles to overcome in order to promote recovery. We have only recently come to understand that the environment of the CNS itself impedes regeneration following SCI (Yiu & He, 2006). Several reports have shown that despite the inhibitory environment, even the adult nervous system is able to make changes (e.g. sprouting of lesioned or spared fibers above (Fouad et al., 2001) and below (Weidner et al., 2001) the spinal lesion) following injury. Enhancing this spontaneous injury induced plasticity to promote recovery in animal models is the topic of this thesis.

In the clinical setting, patients are most often entered into rehabilitation training once they have physically recovered from the trauma of injury itself and the accompanying spinal shock. Though this treatment is generally accepted, there are several unknowns remaining including the best method of training, the amount of time that should be spent in training and the most beneficial delay between injury and training (i.e. is it better to train early after an injury or is it better to wait?). It is also important to remember that to perform treadmill training with patients suffering from SCI, several physiotherapists are needed at a time. This work is hard and incredibly labour intensive and may actually cause injury to the therapist (Wirz et al., 2005). Therefore, there are many issues in the field of rehabilitation training including the manpower needed to do the training and also the methods of training.

Detailed below are the findings from my two projects attempting to promote both plasticity and ultimately functional recovery in rats with SCI. In the first report (Chapter 2; Figure 1.2), the neurotrophins BDNF and NT-3 were used to increase the sprouting of

injured CST axons onto propriospinal interneurons in order to circumvent the lesion and enhance the formation of new circuits. In the second study (Chapter 3; Figure 1.3), grasping training was used to enhance post-injury plasticity while promoting functional recovery following a SCI affecting a forelimb. The two studies are distinct from one another in that the injuries are at different levels in the spinal cord (thoracic for Chapter 2, and cervical for Chapter 3), the treatments are different (neurotrophins application for Chapter 2 and exercise in Chapter 3) and the final anatomical/physiological assessments are also different (CST sprouting onto descending propriospinal interneurons in Chapter 2, and CST sprouting and changes within the sensory motor area of the cortex in Chapter 3).

1.6 Figures

Figure 1.1

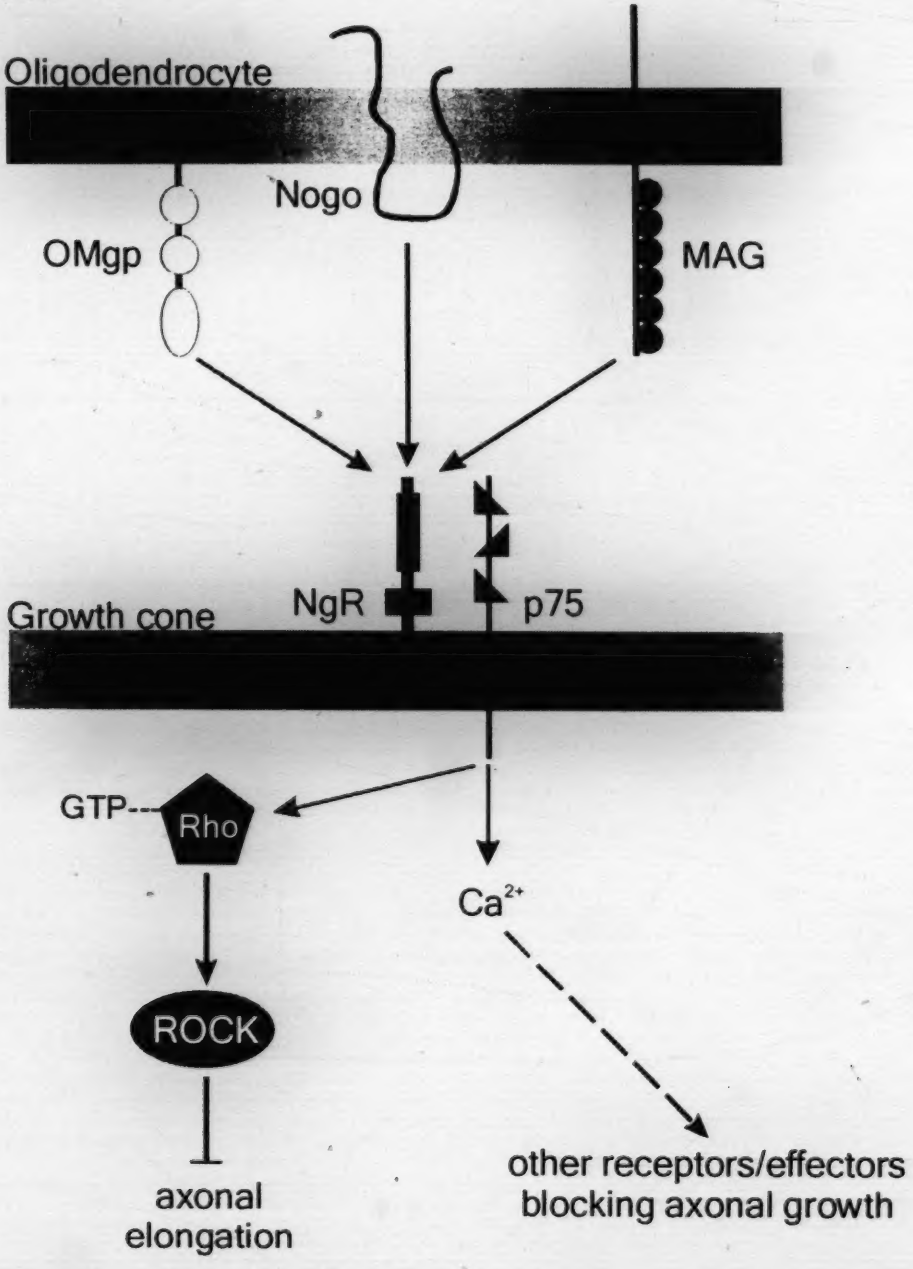

Figure 1.1. Schematic of the inhibitory environment of the CNS. All 3 myelin based growth inhibitors (Oligodendrocyte myelin glycoprotein (OMgp), Nogo and myelin

associated glycoprotein (MAG)) bind to the Nogo receptor (NgR). One of the co-receptors for NgR (p75) is needed to transduce the signal further. Increased intracellular Ca2+ levels are seen following ligand binding to NgR. This has effects on many other receptors and effectors blocking axonal growth (but these are beyond the scope of the current thesis). Activation of NgR also causes an increase in Rho activation. Activation of ROCK then works to inhibit axonal growth/elongation by inhibiting actin reorganizations/promoting collapse.

Figure 1.2

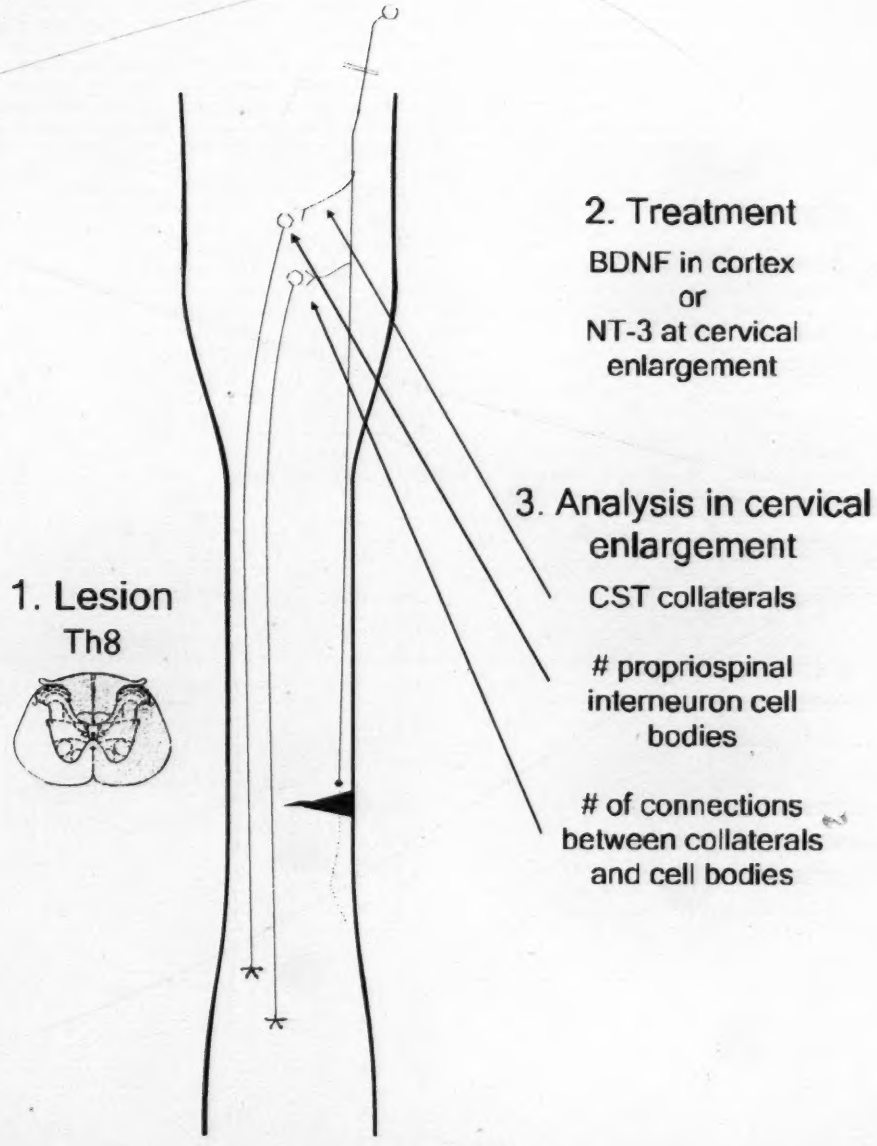

Figure 1.2. Schematic of the study from Chapter 2. Neurotrophins were used to assess their effects on both sprouting of the CST and the number of connections found between these sprouts and long descending propriospinal interneurons in the cervical enlargement, following a partial mid-thoracic injury.

Figure 1.3

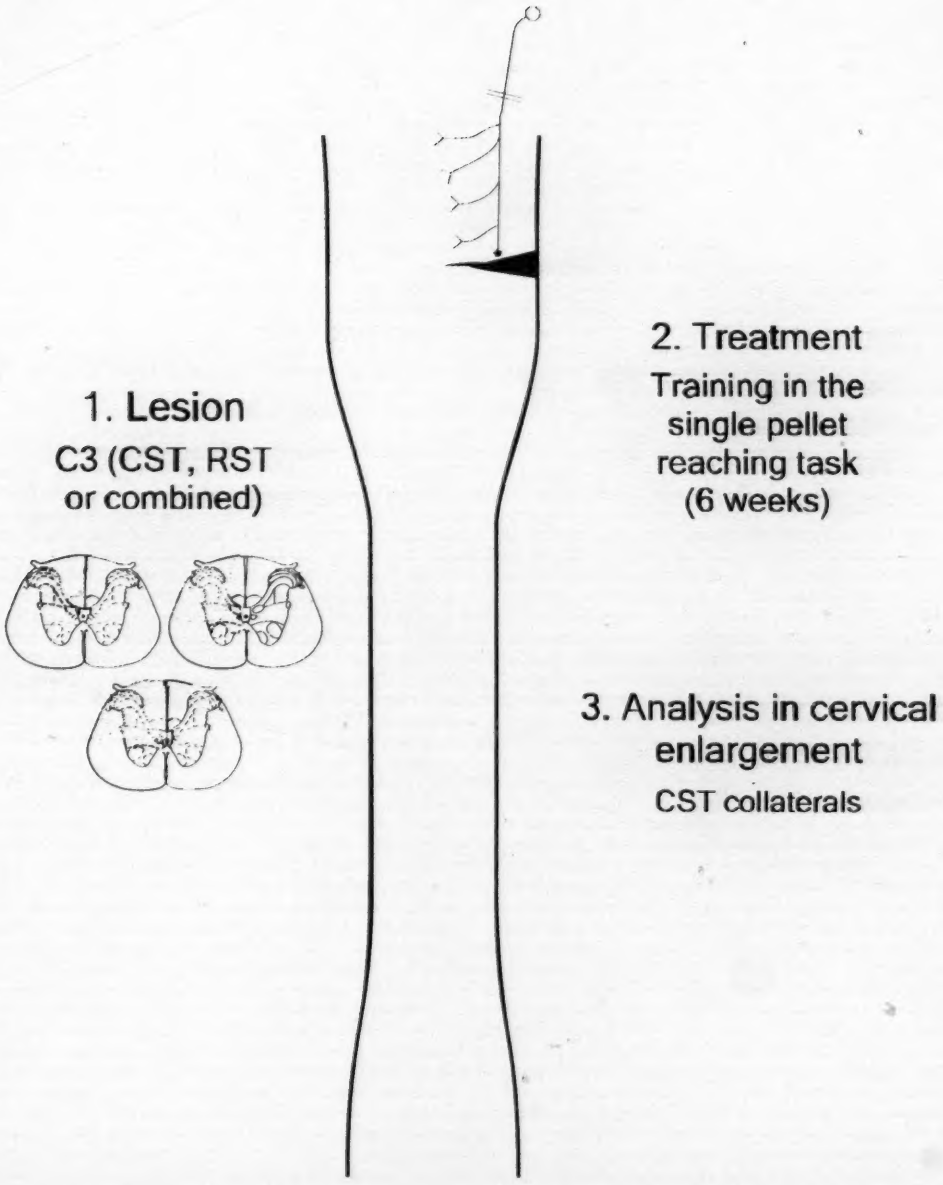
1. Lesion
C3 (CST, RST or combined)
2. Treatment
Training in the single pellet reaching task (6 weeks)
3. Analysis in cervical enlargement
CST collaterals

Figure 1.3. Schematic of the study from Chapter 3. Exercise was used as a treatment following partial SCI at the cervical level. The number of CST collateral sprouts were assessed in the cervical enlargement.

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CHAPTER 2

BDNF PROMOTES CONNECTIONS OF CORTICOSPINAL NEURONS ONTO SPARED DESCENDING INTERNEURONS IN SPINAL CORD INJURED RATS

Adapted from:
Vavrek R, Girgis J, Tetzlaff W, Hiebert GW, Fouad K.
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2.1 INTRODUCTION

Recovery following spinal cord injury (SCI) is limited because severed axons of the central nervous system (CNS) fail to regenerate (Schwab and Bartholdi, 1996). This failure of spontaneous regeneration is due to several inhibiting factors in the injured spinal cord including myelin based inhibitors in damaged myelin and oligodendrocytes and the actual scar formed around the lesion (Yiu & He, 2006). Although regeneration can occur with various therapeutic strategies aimed at neutralizing the inhibitory environment of the spinal cord white matter (e.g. antibodies towards myelin based inhibitors (Liebscher et al., 2005) or digestion of the scar surrounding the lesion; (Bradbury et al., 2002; Fouad et al., 2005) or changing the intrinsic properties of axons and causing them to ignore the inhibitory environment (e.g. cAMP treatments; Nikulina et al., 2003), robust regeneration (i.e. longer than a few millimeters) is rarely seen.

Nevertheless, some recovery of sensory and motor function will occur over the first few weeks following incomplete injuries. This recovery may be attributed to several mechanisms. First, an initial phase of recovery a few days after the injury results from the re-establishment of transmission in uninjured axons, which was blocked during the period of spinal shock (Ditunno *et al.*, 2004; Hiersemenzel *et al.*, 2000; Holaday and Faden, 1983) and the return of motor neuron excitability (Bennett *et al.*, 2004). Second, a later phase of recovery is attributed to re-myelination of spared axons (Jeffery and Blakemore, 1997). Third, long term recovery may occur, including the acquisition of compensatory movement strategies, but the mechanisms involved in this acquisition are not clearly understood (Grasso *et al.*, 2004; Helgren and Goldberger, 1993; McKenna and Whishaw, 1999). They may include axonal sprouting and synaptic rearrangements in spared

neuronal circuits rostral and caudal to the lesion (often referred to as plasticity). Recent studies have shown that the adult CNS is indeed capable of injury-induced plasticity accompanied by moderate functional recovery (reviewed in Ivanco and Greenough, 2000; Raineteau and Schwab, 2001). Examples of such plasticity include the reorganization of cortical maps following brain (Bury and Jones, 2002; Jones and Schallert, 1994), peripheral nerve (Wu and Kaas, 1999) and spinal cord injuries (Bruehlmeier *et al.*, 1998; Fouad *et al.*, 2001; Turner *et al.*, 2003) in humans and animal models. In addition to cortical plasticity, growth of collaterals arising from injured and spared corticospinal tract (CST) fibers has been found following SCI in rats rostral (Fouad *et al.*, 2001), and caudal (Weidner *et al.*, 2001) to the lesion. CST collaterals rostral to the lesion were reported to connect onto spared propriospinal interneurons (PrI), to form new intra-spinal circuits (Bareyre *et al.*, 2004).

Long descending PrIs project from the cervical enlargement to the lumbar enlargement (Miller et al., 1998). They project from the lower cervical/upper thoracic segments (with cell bodies in the intermediate laminae), through the middle of the lateral funiculus to terminate onto interneurons and motoneurons in the lumbar segments of the spinal cord (Jankowska et al., 1974). These fast-projecting fibers are often crossed in the spinal cord between origin and termination (Miller et al., 1998). There are both ascending and descending PrIs, and together, they are thought to function to coordination forelimb-hindlimb locomotion (Miller et al., 1972; Steeves & Jordan, 1980). Since partial lesions of the spinal cord may leave some of the population of descending interneurons intact, they represent a meaningful target for the collateral sprouting found following partial SCI (Jordan & Schmidt, 2002).

The mechanisms promoting injury-induced plasticity at the cellular level are not well understood. Progress in gene profiling has indicated that growth associated genes and neurotrophic factors (e.g., NT-3, BDNF) are up-regulated following injury (Di Giovanni *et al.*, 2003; Hayashi *et al.*, 2000; Song *et al.*, 2001). Hence, spinal cord-derived factors could promote sprouting and the subsequent rewiring of existing pathways. Spinal cord applications of BDNF and NT-3, for example, have been reported to promote neuronal survival and sprouting of the lesioned neurons at the injury site (Bradbury *et al.*, 1999; Bregman *et al.*, 1997; Grill *et al.*, 1997; Hammond *et al.*, 1999; Hiebert *et al.*, 2002; Kobayashi *et al.*, 1997; Liu *et al.*, 1999; Schnell *et al.*, 1994; Ye and Houle, 1997). In addition, when BDNF was applied near the cell bodies it prevented neuronal death and/or atrophy of corticospinal or rubrospinal neurons (Giehl and Tetzlaff, 1996; Hammond *et al.*, 1999; Kobayashi *et al.*, 1997) and promoted regenerative sprouting of injured CST axons (Hiebert *et al.*, 2002). Here, we investigated whether sprouting of CST axons and their connection onto PrI rostral to an injury could be enhanced by applying BDNF intraparenchymally near corticospinal neuron cell bodies or NT-3 intrathecally to the rostral (cervical) spinal cord.

2.2 Materials and Methods

2.2.1 Animals

Experiments were conducted using adult female Lewis rats (Charles River, 180-200g) that were kept at a 12:12 h light dark cycle with water and food provided *ad libitum*. This study was approved by the local animal welfare committee and complies with the guidelines of the Canadian Council on Animal Care.

We examined 7 experimental groups: controls (animals that received no spinal cord lesion and no treatment, n=7), uninjured rats that received vehicle solution to the cortex (n=3), uninjured rats that received BDNF to the cortex (n=3), rats with a thoracic (Th) SCI (details given below) that received vehicle to the cortex (n=6), rats with a Th SCI that received BDNF to the cortex (n=8), rats with a Th SCI that received vehicle solution at the cervical enlargement (n=3), and rats with a Th SCI that received NT-3 to the cervical enlargement (n=9).

2.2.2 Lesions

All operations were performed under Hypnorm (Fentanyl/Fluanisone; Janssen Pharmaceutics, Beerse, Belgium; 120µl per 200g body weight) and Midazolam (Sabex, Canada; 0.75 mg in 150 µl/200g body weight. 750µl total volume diluted with H_2O) anaesthesia. Eye lubricant (Tears naturale®, Alcon Canada, Inc) was applied to protect the eyes from dehydration.

A laminectomy of half of one vertebral lamina at Th_8 was followed by a right-sided dorsal over-hemisection using a customized micro-blade. This lesion ablated approximately 70% of the spinal cord and spared mainly the left ventrolateral quadrant. The surgeon was blinded to the subsequent treatment of the animals. Following the lesion procedure the dorsal back musculature was sutured in layers and the skin closed with surgical clips. Using this lesion rather than a dorsal hemisection (as used in earlier studies by Bareyre *et al.*, 2004; Fouad *et al.*, 2001) we anticipated easier anatomical evaluation due to a lower number of retrogradely labeled interneurons. This lesion also allowed the characterization of the projection pattern of the propriospinal interneurons and focus on

commissural interneurons. Sprouting on a defined population of interneurons like the commissural had not been studied before.

After surgery the animals were kept on a thermostatically regulated heating pad until completely awake. The analgesic Buprenex (Buprenorphine; Reckitt & Colman, Richmond,VA. USA) was administered subcutaneously (0.03 mg/kg) immediately after operation, and every eight hrs for 72 hrs. Ringer solution (4 ml) was given subcutaneously daily for the first week and at later stages if animals showed signs of dehydration.

2.2.3 Neurotrophin Application

During the same operation as the lesion procedure, animals were randomly divided into the different experimental groups and received either BDNF (donated by Regeneron Pharmaceuticals/Amgen) or vehicle to the left sensory-motor cortex, or NT-3 (donated by Regeneron Pharmaceuticals) or vehicle delivered to the cervical spinal cord.

The BDNF or vehicle application to the left motor cortex was performed as described earlier (Hiebert *et al.*, 2002). In brief, a hole in the cranium was made at coordinates 2.0mm lateral and 1.5mm caudal from Bregma. A cannula from the Alzet brain infusion kit (Durect Corporation, Cupertino, CA) was inserted 1.5mm into the motor cortex. Two stainless steel screws inserted into the parietal plates were used to secure the cannula using dental acrylic. The infusion cannula was connected to a 14 day Alzet osmotic mini-pump (0.5µl/hr) that was placed subcutaneously in the back. The osmotic pump was filled with either vehicle solution (0.25% rat serum albumin in 0.1M phosphate buffered saline) or BDNF (1µg/µl in vehicle).

The application of NT-3 or vehicle to the cervical enlargement was performed by the intrathecal insertion of a fine catheter (Recathco, LLC, Allison Park, PA) at the cisterna magna as described earlier (Yaksh and Rudy, 1976). The catheter tip was inserted subdurally and gently moved until it reached the C3 segment (determined by measuring both the catheter length and the length of spinal segements where the catheter was inserted). The catheter was connected to an Alzet mini-osmotic pump (0.5µl/hr) that was placed subcutaneously in the back and filled with either vehicle (see above), or NT-3 (1µg/µl in vehicle). The pump and catheter were removed after two weeks.

2.2.4 Behavioral testing

One week before the lesion operation the animals were trained to walk on a 1m long horizontal ladder with metal rungs, elevated 30cm from the ground. A testing session consisted of 3 ladder passes and performance was captured using a digital video camera (Canon, Optura 100, 60 fields/s). The analysis involved counting the number of errors in placing of the right hind limb (the side of the spinal cord that has been in most animals completely ablated) and averaging errors from the 3 passes for each animal. An error was defined as a slip off of a rung or a fall between rungs made by the right hind limb. A defined 10-bar sector was chosen for analysis. To prevent habituation to a fixed bar distance, the bars in this sector were placed irregularly (1-3cm spacing) and were changed for each testing session. The animals' performance was monitored weekly up to 4 weeks after surgery. Animals were excluded from behavioral testing if they were not able to walk on the horizontal ladder (>8 mistakes/trial).

2.2.5 Tracing

Four weeks after lesion (2 weeks after removing the mini-pumps), retrograde tracing of propriospinal interneurons projecting into the lumbar enlargement was achieved by bilateral injection of 1µl of 5% FlouroGold (Chemicon International, Inc Temecula, CA) into the L2 segment of the spinal cord. This retrograde tracer is taken up by injured axons and/or nerve terminals in the proximity of the injection site. Under anesthesia (see above) rats were held in a stereotaxic device and a laminectomy of half of the T12 vertebral segment overlying the L2 spinal segment was performed. The lumbar vertebra was lifted using tissue forceps mounted onto a micro-drive to stabilize the spinal cord. A microcapillary (glass electrode) with an approximate tip diameter of 20-30µm was mounted onto a 5µl Hamilton syringe and lowered into the exposed spinal cord about 200-300µm lateral to the midline (depending on the location of blood vessels) and to a depth of 400µm. The capillary remained in place for 1 minute following the injection.

In the same surgery as the retrograde tracing, the corticospinal tract was anterogradely labeled in the left cortex (the side that projects into the right side of the spinal cord) using a 10% solution of biotinylated dextran amine (BDA 10,000MW, Molecular Probes, Invitrogen Corporation, Burlington, Ontario). Using a glass microelectrode mounted onto a Hamilton syringe 1µl of BDA was slowly (over 3min) injected into the hind limb area of the motor cortex (2mm lateral and 1.6mm caudal to Bregma, at a depth of 1.5mm). The electrode remained in place for another 3 minutes following the injection.

Two weeks following the tracer injections (to allow the BDA to reach its target) the rats were perfused transcardially with a Ringer's solution containing 100 000 IU/l

heparin, followed by 4% paraformaldehyde solution in 0.1M PB with 5% sucrose as fixative. The spinal cords and brains were removed, post-fixed overnight in 4% formaldehyde and then transferred to a 30% sucrose solution for 3 days. Thereafter, the spinal cords and brains were cut into 5 sections (brain, C1, cervical enlargement, upper thoracic (Th2-7), and Th7-9) embedded with Tissue Tek (Sakura Finetek USA Inc, CA) and frozen at minus 60° Celsius.

2.2.6 Anatomical analysis

Using a cryostat, 25µm sections were taken through different areas of the CNS. Sagittal sections of the brain were taken at the BDA injection site. Cross-sections of the C1 segment were taken to count the total number of traced CST fibers. The cervical enlargement (C3-6), as well as the upper thoracic spinal cord (Th2-7) were sectioned horizontally to quantify CST collaterals, PrI, CST collateral contacts with PrI, and CST collateral projections. Cross-sections through the lesion at Th7-9 were used to analyze lesion size. Finally, cross-sections of the C1 segment were used to analyze the effects of NT-3 application onto CGRP (see below) positive fibers. Unbiased blind analysis of the tissue was ensured by number coding of the slides.

Staining for BDA was performed according to earlier reports (Fouad *et al.*, 2001). Slides were dried in an incubator at 38° Celsius for 1h and washed twice for 10 minutes in 50 mM Tris buffered saline (TBS), pH 7.4, followed by two 45 min washes with TBS containing 0.5% Triton X-100. Afterwards the slides were incubated overnight with an avidin-biotin-peroxidase complex in TBS with Triton (ABC elite, Vector Labs, Burlingame, CA) according to the instructions of the manufacturer. Subsequently the

DAB reaction was performed using the Vector DAB kit (SK4100, Vector Labs). The reaction was monitored and stopped by extensive washing in water. FlouroGold was detected on the same slides using primary FlouroGold antibodies (1:1000 Chemicon), incubated overnight at 4° Celsius followed by washing in TBS. The secondary antibody (Vector) was incubated overnight at 4° Celsius and visualized with the Vector Nova Red kit. The slides were dehydrated with alcohol and cleared with xylene and coverslipped in Permount (Fischer Scientific).

Evaluation of BDA injection site: To confirm that the BDA injection in the cortex was restricted to the hind limb area, the cortices were sectioned in the sagittal plane, stained for BDA (see above) and spreading of BDA measured at the injection site. BDA did not spread more than 1.5mm from the injection site in any of the animals included in this study (thus the tracer did not approach the fore limb area).

Counting traced CST axons: To avoid spread of the tracer into cortical fore limb areas, BDA was only injected at one position with a fairly low volume. This resulted in low numbers of traced fibers, thus allowing precise counting. Quantification of the total number of traced CST axons rostral to the injury was performed on C1 cross-sections using light microscopy (40x). Pictures were taken using a digital camera mounted on a Leica microscope, and a grid overlying the pictures was used to assist counting of the traced axons.

Counting of PrI and contacts between CST collaterals and PrI: Nova Red stained PrI cell bodies were counted on the same horizontal sections used for collateral analysis (see

below). A contact was scored when a bouton-like structure of a BDA positive fiber was co-localized with the soma or dendrite of a NovaRed stained PrI (Fig.2.5A). Normalization of these contacts was performed comparable to Bareyre *et al.* (2004). Therefore, the counted number of contacts was divided by both the number of PrI (to account for variability in the retrograde tracing) and the number of collaterals (to account for the variability of collaterals), or the number of PrI and the total number of CST fibers (Normalized contacts = counted # of contaets/PrI/collaterals, or = counted # of contacts/PrI/total # of traced CST fibers).

Counting of CST collaterals: Horizontal sections of the cervical enlargement and the thoracic spinal cord (rostral to the injury) were cut for the quantification of CST collaterals. Every third section was analyzed. Collaterals emanating from the traced CST and crossing into gray matter for more than 30 µm over an approximated line drawn at the white/gray matter interphase were counted under light microscopy (Fig.2.3). The length (in mm) of the analyzed section was measured and used to normalize the counted collaterals. Finally, as described in Raineteau *et al.* (2001), the collaterals were expressed as the number of collaterals divided by both, the length analyzed and by the total number of traced CST fibers (Normalized collateral value = #Collaterals/mm/total # of traced CST fibers).

To ensure that the number of counted CST collaterals was not influenced by BDNF induced changes in their diameter we took pictures of 5 collaterals/animal using oil immersion light microscopy (100x). The collateral diameter was then measured with the Scion Image (NIH) program.

Projections of CST collaterals: This analysis was performed on ventral sections of the same set of horizontal sections of the cervical spinal cord that were used for the analysis of collaterals and contacts. We divided the spinal cord with an approximated line (indicated in figure 2.6A), separating the gray matter of the right side into a medial and a lateral part. The lateral side contains among others the motoneuron pool innervated by the CST (Kuchler *et al.*, 2002), and the medial side contains among others PrI. BDA positive CST fibers were counted within these areas using light microscopy (40x).

Analysis of lesion size: Lesion size was analyzed on a subsequent series of cross-sections of the Th7-9 spinal segment throughout the lesion. The sections were counterstained with 0.1% Cresyl Violet. Pictures at the maximal lesion size were taken (10x) and reconstructed. The amount of spared white matter was determined using the Scion Image (NIH).

CGRP staining and density measurement: As a positive control for the NT-3 application we stained CGRP positive fibers on cross-sections of the C2 segment only in the NT-3 treated or NT-3 control group (Hagg *et al.*, 2005). The primary antibody to CGRP (Chemicon) was detected with a biotinylated secondary antibody (PK 6101 Vectastain; Vector Laboratories) and visualized with an avidin-biotin complex (Vector Laboratories) followed by DAB. Digital images were captured using light microscopy (10x). The extent of the DAB-labeled CGRP fibers in the dorsal horn was quantified in a blinded manner using Scion Image analysis software (NIH), which was calibrated to perform area

measurements in mm^2. A boundary encompassing the entire dorsal horn was drawn on the image and the region within this boundary comprised the area to be quantified. CGRP immunoreactivity within the designated area was identified by pixel thresholding, which converts areas of immunoreactivity into black pixels on a white background. Analysis was performed by using the average density measure of 5 sections/rat.

2.2.7 Statistics

Statistical comparisons were performed using Prism (GraphPad software, San Diego, CA). Comparisons between different groups were made using unpaired *t*-tests. Comparisons of PrI numbers on the left and right side of the spinal cord were made using a paired *t*-test. In cases where the data did not follow a normal distribution (i.e., projections into gray matter did not pass a Kolmogorov-Smirnov test) non-parametric tests were used (Mann-Whitney U tests), preceded by a Kruskal-Wallis test.

The correlation between number of contacts and errors on the horizontal ladder were performed using the Pearsons' correlation coefficient. Differences with $p<0.05$ were considered significant. Error bars are given as standard error of the mean.

2.3 Results

2.3.1 Most propriospinal interneurons linking the cervical and lumbar enlargements are commissural

The first reports on injury-induced sprouting of transected CST axons rostral to a spinal cord lesion (Bareyre *et al.*, 2004; Fouad *et al.*, 2001) studied the effects of a dorsal lesion (hemisection). In the present study we performed a right sided over-hemisection,

which ablated the dorsal columns and dorsolateral funiculi on both sides, and severed the right lateral and ventral columns either completely or with minor sparing (<10%). The left ventrolateral funiculus was spared in all the animals. We found that the retrograde tracer FlouroGold injected into the lumbar enlargement was taken up by spared propriospinal interneurons (PrI) with cell bodies in both sides of the thoracic and cervical spinal cord. The cell bodies were arranged in a fairly organized fashion, like beads on a string, lateral to the central canal in medial portions of laminae VII and VIII (Fig.1). When comparing the numbers of cell bodies in the left and right side of the cervical spinal cord in lesioned animals, we found that a larger proportion of PrI crossed the midline rostral to the injury site. This is based on the finding that although the right side of the spinal cord was lesioned, 60% of the PrI cell bodies in the cervical enlargement (C3-7) were labeled on the right side. Considering that the vast majority (in most cases the entire side) of the right spinal cord was ablated, most of these neurons must be commissural interneurons that cross the midline rostral to the injury (Fig.2.2A,B). The number of cell bodies in the cervical enlargement on the lesioned (right) side of the BDNF treated rats was on average 253±45 (SE) as compared to 186±48 PrI on the left side, which apparently did not cross. This significant ($p=0.007$) left versus right difference stands in contrast to the evenly distributed cell numbers in unlesioned control rats (410±83 versus 395±85), demonstrating that the results were not due to asymmetric tracer injections. While we can not rule out that some axons of PrI may undergo multiple midline crossings above or below the lesion, the net effect reveals that about 40% of the PrI axons are uncrossed and 60% crossed. Following our lesion model the number of PrI

in lesioned animals is approximately half that of unlesioned animals, confirming that half of the cell population has been axotomized and hence unable to take up the tracer.

The ratio of crossing and non-crossing PrI in the cervical enlargement stands in contrast to the 50:50 ratio in the thoracic spinal cord (Fig.2.2C,D). When counted at Th 3-7 the total number of PrI was not statistically different to those found in the cervical enlargement, however, the ratio between crossing and non-crossing fibers was equal. In lesioned rats we found 241±85 on the right versus 239±79 on the left. As expected, there was no difference in the numbers of PrI between the two sides within the thoracic spinal cord of unlesioned (control) rats (360±42 on the right and 378±47 on the left).

2.3.2 Only BDNF application together with injury increases collateral sprouting at the cervical level

Earlier studies have shown that collateral sprouting of severed CST axons can occur rostral to an injury, possibly as an adaptive process to promote functional recovery after spinal cord injury (Bareyre *et al.*, 2004; Fouad *et al.*, 2001). Since we have shown previously that BDNF application to the cell bodies of CST neurons promoted regenerative sprouting of their axons (Hiebert *et al.*, 2002) we hypothesized that application of BDNF (12µg/day) at the cell body would also enhance the collateral sprouting of CST axons onto cervical PrI after a thoracic overhemisection. Additionally, based on earlier reports showing a response of sensory axons (Bradbury *et al.*, 1999) and CST (Schnell *et al.*, 1994) axons to local NT-3 application (12µg/day), we assessed the effect of NT-3 given locally to the cervical enlargement.

We counted the number of collaterals, emerging from the traced CST fibers into the gray matter of the cervical spinal cord on the lesioned (right) side. These counts were normalized to the total number of traced axons counted at C1, and the distance evaluated (Fig. 2.3, original numbers are presented in Table 2.1). In contrast to reports with dorsal lesions only (Bareyre *et al.*, 2004; Fouad *et al.*, 2001), we found no increase in CST collaterals in lesioned vehicle treated animals, compared to unlesioned animals (Fig.3B). However, the application of BDNF to the cell bodies in the motor cortex significantly ($p=0.037$) increased the normalized number of CST collaterals (Fig. 2.3B). This stands in contrast to the BDNF application to the cortex in unlesioned rats, where no change in the number of collaterals was found. Note that the normalized collateral number in unlesioned animals is not higher than in those with lesions, indicating that the increase in collaterals in the BDNF group cannot be explained by sparing of axons on the right side in some of the rats.

To ensure that the quantification of collaterals following BDNF application was not biased by an effect of BDNF on the collateral thickness, we measured and compared the diameter of collaterals between animals that received vehicle or BDNF to the cortex ($0.75\mu m \pm 0.03$ and $0.74\mu m \pm 0.01$ respectively). There was no statistical difference between the two groups.

The local NT-3 application to the cervical enlargement did not promote collateral sprouting of the CST (Fig. 2.3C) and no statistical difference between the groups was found. To verify that the application of NT-3 was effective, we examined the effect on CGRP positive fibers in the dorsal horn (Fig. 2.4). Similar to the previous report by Hagg *et al.* (2005), we found a significant ($p=0.045$) increase in fiber density (pixilated

area/mm^2) in the NT-3 treated rats (92.3±1.3 as compared to 85.9±0.7 in vehicle treated rats).

2.3.3 Bouton-like structures of CST collaterals contact propriospinal interneurons

Bareyre *et al.* (2004) described CST collaterals contacting long descending PrI and thus circumventing the lesion in order to connect to the distal cord. We therefore hypothesized that the pharmacologically induced collaterals of lesioned CST fibers are likely to contact interneurons in the cervical enlargement rostral to the lesion. To examine this we counted the occurrence of bouton-like structures of collaterals emanating from the BDA traced CST, that were co-localized with somata or dendrites of retrogradely traced PrI (FlouroGold, Fig. 2.5A). We define these occurrences as contacts, without implying that these are necessarily synaptic contacts. These contact counts were normalized to the number of traced CST fibers and the number of labeled PrI (Fig. 2.5B). Alternatively, to account for the increased number of collaterals, the number of bouton like structure/PrI contacts was normalized to the number of collaterals (instead of the total number of traced fibers) and the number of PrI (Fig. 2.5C). The total numbers of counted contacts, PrI and number of collaterals are presented in Table 1. When normalized to the number of traced axons and the number of PrI, we found that the number of contacts did not increase following injury alone, or BDNF application without injury (Fig. 2.5B). This is consistent with our finding that the number of CST collaterals did not increase in these groups. A small and statistically not significant increase was seen following local NT-3 application. However, following BDNF application to the cell bodies of injured animals, the number of contacts was significantly increased by 287% ($p= 0.037$), when normalized

to the number of traced fibers and compared to lesioned and vehicle treated animals. When normalized to the number of collaterals there was a statistically insignificant increase of 229% ($p=0.13$; Fig. 2.5B, C). Hence, the increased number of contacts after BDNF treatment is largely due to more collaterals that are forming contacts and to a lesser extent to more contacts per collateral.

2.3.4 BDNF induced collaterals are not specifically targeting PrI

Following spinal cord injury reorganization of the motor cortex has been reported in humans and animal models (Bruehlmeier *et al.*, 1998; Cohen *et al.*, 1991; Fouad *et al.*, 2001; Green *et al.*, 1999; Lotze *et al.*, 1999). A possible mechanism may involve projection of the injured CST fibers (that normally project to the lumbar spinal cord) to alternative targets, such as motoneurons of the fore limb. In this part of the experiment we wanted to know if injury and BDNF-induced CST collaterals are specifically targeting PrI located in the medial parts of Lamina VII and VIII, or whether they also grow towards the fore limb motoneuron pool that we have described earlier (Kuchler *et al.*, 2002). As NT-3 application did not increase collateral sprouting we excluded the NT-3 groups from this analysis. We divided the sections with an approximated line (indicated in figure 2.6A), which separated Lamina VII and VIII in a medial part (containing PrI) and a lateral part (containing among others the motoneuron pool). As the density of BDA positive projections in the cervical gray matter was fairly low, individual branches (segments of BDA positive projections) could be counted in the medial and lateral part. We found that the BDA positive projections within the medial gray matter doubled, in contrast to no detectable changes in collateral counts and number of contacts to PrI

following spinal cord lesion and vehicle treatment. This increase which could be due to increased arborisations of the collaterals was significant ($p=0.035$), when compared to uninjured rats (Fig. 2.6C). The number of BDA positive projections in the lesioned BDNF treated group increased even further and the difference was significant, when compared to unlesioned ($p=0.003$) and lesioned vehicle treated rats ($p=0.029$). When quantifying CST projections within the lateral areas, we found similar increases as in the medial areas. The counts in the lesioned vehicle treated group increased 13.8 times when compared to the unlesioned controls, however this increase was not significant ($p=0.1$). BDNF application in injured rats significantly increased the numbers by 2 orders of magnitude when compared to unlesioned rats ($p=0.0003$) and about 7 times compared to the lesioned vehicle treated rats ($p=0.012$). Thus, BDNF induced sprouting is not exclusively targeted to PrI in the medial spinal cord.

2.3.5 Neither injury alone nor the addition of BDNF increase sprouting of injured CST fibers at thoracic level

Cell bodies of PrI with spared axons (FlouroGold positive) could be found within the entire spinal cord rostral to the lesion. In this part of the experiment, we wanted to examine whether increased collateral sprouting caused by BDNF application was localized only to the cervical enlargement or whether it also occurred in the upper thoracic spinal cord, directly rostral to the injury (Th8). We did not find significant changes in the number of CST collaterals in either the lesion vehicle treated or the lesion and BDNF treated group (Fig. 2.7A). As NT-3 was applied locally at the cervical level

and did not yield any effects on the CST collaterals at that level, we did not further analyze the spinal cord tissue at the thoracic level.

At the thoracic level, there was a non-significant trend towards increased numbers ($p=0.4$) of contacts between CST collaterals and PrI, following lesion when compared to uninjured rats without vehicle. Following BDNF application an additional 2 fold (non-significant) increase was found (Fig. 2.7B).

2.3.6 Increase in CST-PrI contacts correlates with functional recovery

A major question raised by the findings of BDNF-promoted sprouting and rerouting of CST fibers is the functional relevance of these anatomical changes. Seeking a behavioral correlate, we pooled the number of cervical CST-PrI contacts (normalized to the total number of traced axons and the number of PrI) of lesioned vehicle treated versus lesioned plus BDNF treated rats and plotted these results against the error rate when walking across a horizontal ladder (grid walk). The result was a significant correlation with an r value of 0.71 shown in figure 2.8. Although the BDNF treated rats performed on average better (5.6 errors ±1.2) than the lesioned and vehicle treated rats (6.5±0.9), the comparison between the groups was not statistically significant.

2.4 DISCUSSION

In this study we show that the combination of a thoracic spinal cord over-hemisection injury with BDNF application to the cell bodies of the lesioned CST neurons in rats promoted the sprouting and possibly the rewiring of CST axons in the cervical spinal cord. The sprouting of CST axons onto spared propriospinal interneurons could

allow interrupted corticospinal signals to bypass the lesion site and reach the distal cord without the need for axonal regeneration. Recent studies have indicated that the injured CST is able to sprout rostral (Fouad *et al.*, 2001) and caudal (Weidner *et al.*, 2001) to a spinal cord lesion. Importantly, CST collaterals were reported to connect onto PrI rostral to a dorsal hemisection of the thoracic spinal cord (Bareyre *et al.*, 2004). In contrast to the studies by Bareyre *et al.* (2004) and Fouad *et al.* (2001), in which both ipsi- and contralateral projecting interneurons were spared, we did not find increased collateral sprouting or an increased number of contacts of injured CST fibers onto commissural PrI after SCI alone without further treatments. Since the approach of tracing and counting collaterals was comparable to the earlier studies, technical differences to explain the different results are unlikely. However, the lesion in our study was larger than the one used by Bareyre *et al.* (2004) and Fouad *et al.* (2001), because we also axotomized most of the non-crossing PrI. This modified significantly the property of these cells as potential targets for the collaterals since the non-crossing interneurons had been axotomized. Typically axotomized neurons lose many of their synaptic inputs, which would suggest they do not attract sprouts either. Reconciling our previous work with the present study would hence lead to the speculation that the CST spouting observed earlier after dorsal hemisection (Baryere *et al.* 2004, Fouad *et al.* 2001) was mainly onto ipsilaterally projecting interneurons. Our findings that injured CST fibers did not show increased spontaneous contacts onto PrI could thus be explained by the fact that PrI labeled in our experiments will cross the midline (aside from a small number of spared fibers on the right side of a few animals) and therefore do not include this ipsilaterally projecting population. The commissural interneurons might not represent a functional target for CST

fibers that have already crossed at the level of the brain stem. A connection onto these PrI would therefore result in a re-crossing of the pathway. Together, the present results and the results by Bareyre *et al.* (2004), suggest that injury-induced sprouting of CST collaterals and connections on PrI occurs only when non-crossing PrI remain connected to lower spinal cord levels. This is in line with the finding of Baryere *et al.* (2004), who indicated that CST contacts onto short PrI (that do not pass the lesion site) will form, but not persist.

Another interesting finding is that although the cervical, as well as the thoracic, spinal cord contained cell bodies of spared PrI projecting to the lumbar enlargement, collateral sprouting of CST axons and increases in CST to PrI connections only occurred at cervical levels. A possible reason is that the connections between the cervical and lumbar enlargements are especially important as they link networks controlling leg movements (Kjaerulff and Kiehn, 1996), despite the limited contribution of the CST to locomotor control in rats (Muir and Whishaw, 1999; Schucht *et al.*, 2002).

The application of neurotrophic factors and other agents to cell bodies has been examined in various models (Benowitz *et al.*, 1999; Hiebert *et al.*, 2002; Lu *et al.*, 2004; Plunet *et al.*, 2002; Tetzlaff *et al.*, 1994). However, the earlier studies on the descending spinal cord projections where neurotrophins were applied at the lesion site or at the cell body focused on neuroprotection of the cell bodies, gene expression and regenerative growth of lesioned axons (Bregman *et al.*, 1997; Giehl and Tetzlaff, 1996; Grill *et al.*, 1997; Hammond *et al.*, 1999; Kobayashi *et al.*, 1997; Schnell *et al.*, 1994). Here we report that collateral sprouting rostral to an injury with the potential of reconnecting to the periphery via PrI can be promoted by BDNF infusion into the vicinity of the

corticospinal neurons. While such application is clinically not applicable in this present form, this finding represents a proof of principle of a novel treatment opportunity, which is especially important considering the large number of patients presenting with incomplete spinal cord injuries.

Comparable to a recent study by Hagg *et al.* (2005), we found that the local application of NT-3 did not result in increased sprouting of injured CST fibers. This appears to stand in contrast to studies that reported increased spouting of injured CST fibers at the injury site following local NT-3 application (e.g., Schnell *et al.*, 1994) or of spared fibers (contralateral to the lesion) distal to the lesion (Zhou *et al.*, 2003). In both cases there were additional factors involved as for example Wallerian degeneration or freed synaptic space, thus NT-3 on its own may not be sufficient to enhance sprouting.

Our data indicate a non-significant reduction of collaterals following NT-3 application, which appears different from the study by Hagg *et al.* (2005), as they reported a significant reduction. The non-significant reduction in our study could be due to the relatively small number of animals in our lesioned and vehicle only treated control group. Another possibility is that the tissue penetration of NT-3 was very weak, thus resulting in a modest effect only in axons located in the superficial layers but none in the more medial located CST.

Our study compared the functional outcome between the injured, BDNF treated and untreated animals, by their ability to cross a horizontal ladder, a task requiring CST integrity (Metz and Whishaw, 2002). We found a significant correlation between the number of CST collateral contacts to PrI and the performance on the ladder walk. This suggests that new collaterals from the injured CST and their connections onto crossed PrI

might be functionally meaningful. This idea is supported by our earlier finding that following complete CST lesion at the level of the brainstem and neutralisation of Nogo-A, functional rerouting of the CST via the red nucleus was possible (Raineteau *et al.*, 2002; Raineteau *et al.*, 2001). Further studies with detailed electrophysiology and higher animal numbers, as well as other neuronal sprouting enhancing treatments (e.g., anti-Nogo A) will show whether rerouting injured tracts within the spared spinal cord is a viable method to restore function after spinal cord injury.

2.5 Figures
Figure 2.1

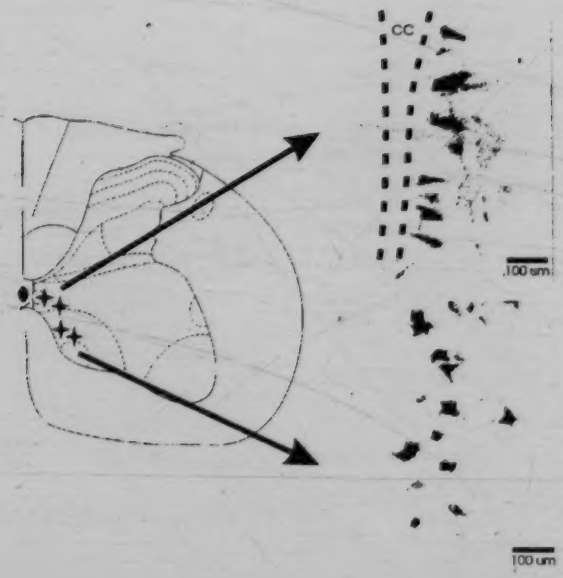

Figure 2.1. Location of PrI within the cervical enlargement following thoracic spinal cord injury. Two populations of PrI were found, one located lateral to the central canal in lamina VII, and the other more ventral in lamina VIII.

Figure 2.2

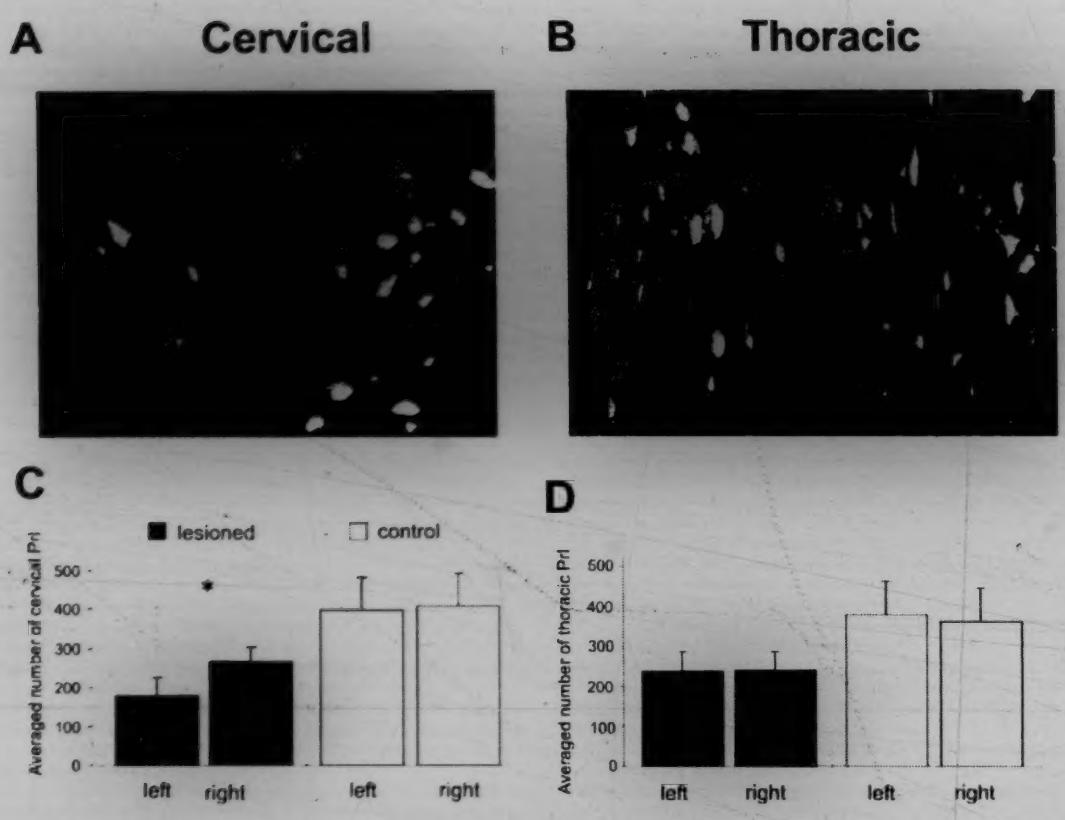

Figure 2.2. Distribution of long descending PrI in the cervical (A,C) and thoracic spinal cord (B,D). Following a spinal cord lesion at Th8 that ablated PrI axons of the right side, we found a significantly higher number of retrogradely traced PrI cell bodies on the right side, as compared to the left (A, C). Thus there are more PrI that project contralateral than ipsilateral. In unlesioned animals the ratio of cell bodies within the left and right spinal cord is even (C). In the thoracic spinal cord in lesioned as well as unlesioned animals the ratio of cell bodies is even (D). Error bars indicate standard error of the mean; * = $p<0.05$.

Figure 2.3

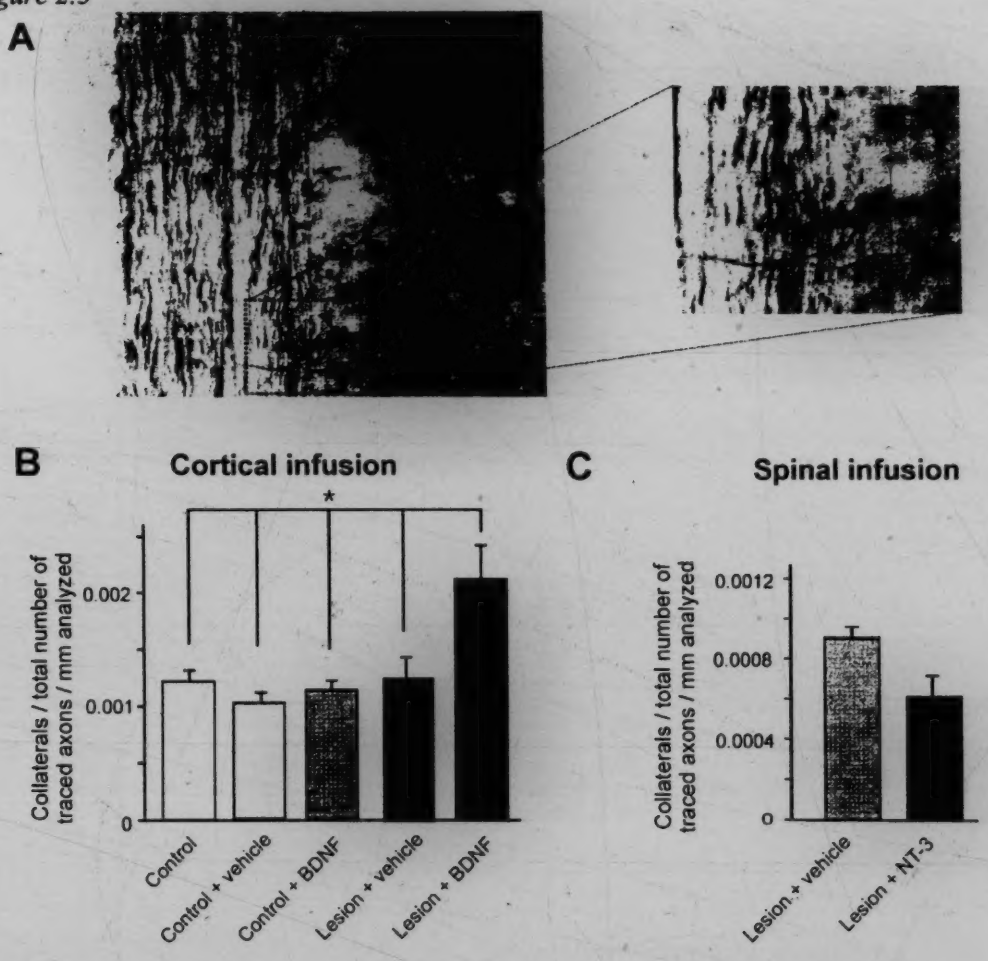

Figure 2.3. Corticospinal tract (CST) collaterals at cervical level. Collaterals arising from the right (lesioned) CST that are entering the gray matter were counted on horizontal sections (A, see arrowheads). The dashed line indicates the white matter-gray matter interface. Following lesion or lesion and the application of NT-3 we did not find an increase in the normalized number of collaterals (B,C). The application of BDNF to lesioned rats, however, significantly increased the number of collaterals. BDNF application to uninjured animals did not show an effect (B). Error bars indicate standard error of the mean; * = $p<0.05$.

Figure 2.4

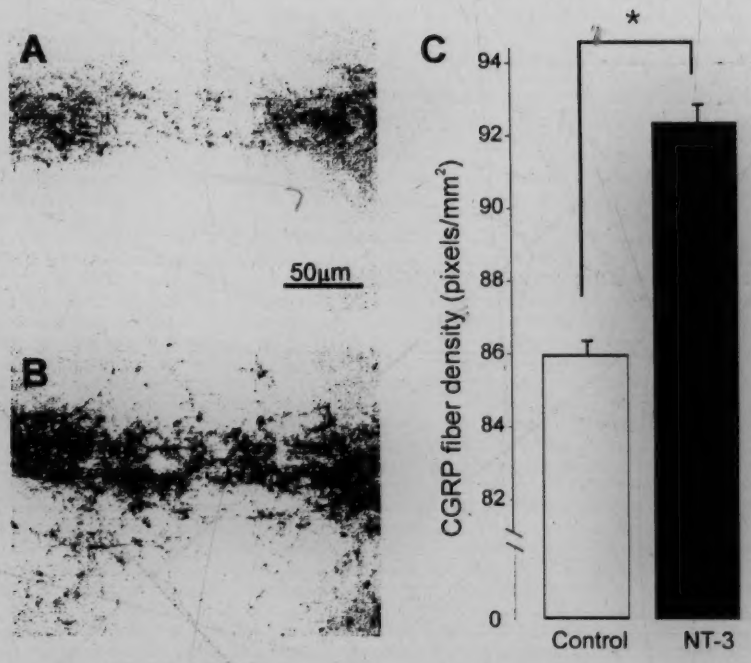

Figure 2.4. Fiber density of CGRP positive terminals in the dorsal horn of the cervical spinal cord in lesioned vehicle treated (A) and lesioned and NT-3 treated rats (B). Using Scion Image software we found a significant increase in fiber density in lesioned and NT-3 treated animals, when compared to lesioned vehicle treated rats (C). Error bars indicate standard error of the mean; * = $p<0.05$.

Figure 2.5

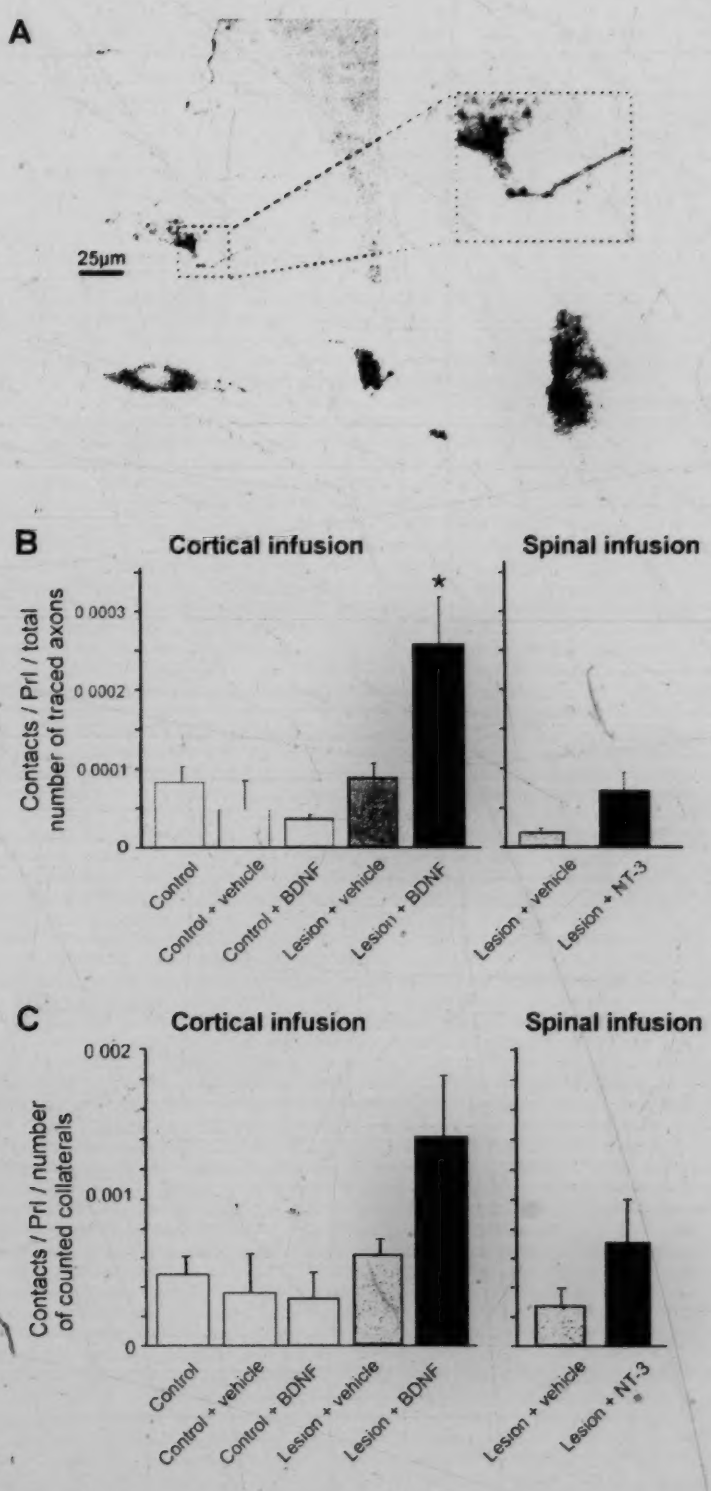
A
25µm
B
Cortical infusion
Spinal infusion
Contacts / PrI / total number of traced axons
0 0003
0 0002
0 0001
0
*
Control
Control + vehicle
Control + BDNF
Lesion + vehicle
Lesion + BDNF
Lesion + vehicle
Lesion + NT-3
C
Cortical infusion
Spinal infusion
Contacts / PrI / number of counted collaterals
0 002
0 001
0
Control
Control + vehicle
Control + BDNF
Lesion + vehicle
Lesion + BDNF
Lesion + vehicle
Lesion + NT-3
*

Figure 2.5. CST – PrI contacts in the cervical spinal cord. We used a combination of anterograde tracing (BDA tracer injection into the hind limb area of the motor cortex) and retrograde tracing (FluoroGold tracer is taken up by axons that project to the lumbar enlargement) to visualize CST fibers and PrI within the cervical gray matter. Examples of co-localizations that qualified as contacts are given (A). After counting the number of contacts of bouton-like structures from CST collaterals and PrI, we normalized these counts to either the total number of traced CST axons counted at C1 and PrI (B) or to the number of counted collaterals and PrI (C). Following normalization to the total number of traced fibers we found no difference between unlesioned, unlesioned and BDNF treated, lesioned and vehicle treated or lesioned and NT-3 treated animals, but a significant increase to all other groups, following lesion and BDNF application. Following normalization to the number of counted collaterals there was still an increase, however, this was not statistically significant. Error bars indicate standard error of the mean; * = $p<0.05$.

Figure 2.6

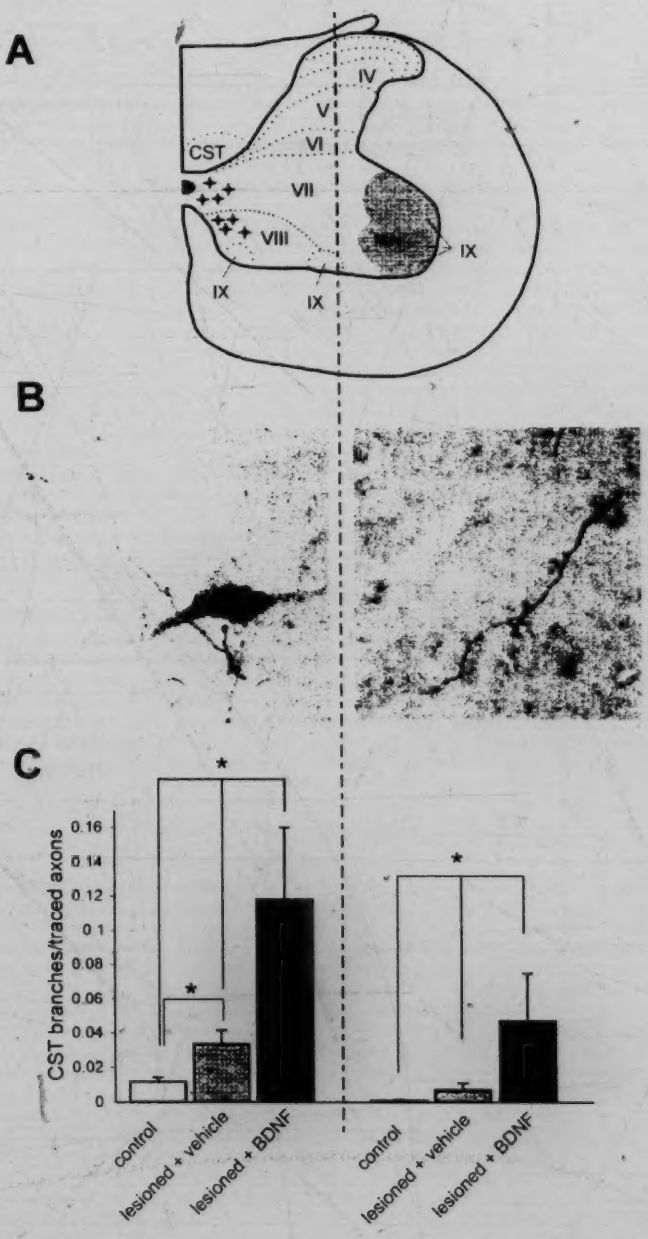
A
IV
V
VI
CST
VII
VIII
IX
IX
IX
B
C
CST branches/traced axons
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
*
*
*
control
lesioned + vehicle
lesioned + BDNF
control
lesioned + vehicle
lesioned + BDNF

Figure 2.6. Projection of CST collaterals into the ventrolateral gray matter of the cervical spinal cord, normalized to the total number of traced fibers. To determine whether CST fibers project exclusively to the location of PrI in the medial gray matter (see stars in A), or whether they also project towards fore limb motoneuron pools (indicated in dark gray), we counted the number of fiber sprouts within the lateral and medial gray matter of Lamina VII and VIII. BDA positive fibers in the medial (left) and lateral gray matter (right) are shown in B. Different to collateral counts entering the gray matter the number of fibers within the medial gray matter is significantly increased following injury and vehicle treatment only (C). The application of BDNF significantly enhanced the lesion effect. BDNF application also significantly increased the projections of CST fibers into the lateral gray matter where motoneuron pools are located. Error bars indicate standard error of the mean; * = $p<0.05$.

Figure 2.7

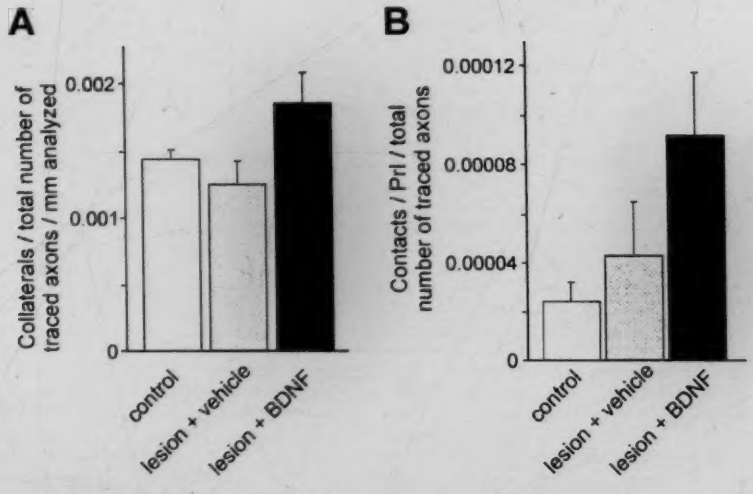

Figure 2.7. CST collaterals (A) and their contacts to PrI (B) at thoracic level. When performing the same analysis as at the cervical enlargement, there were moderate but not significant changes found in the number of collaterals or contacts between CST fibers and PrI. Error bars indicate standard error of the mean; * = $p<0.05$.

Figure 2.8

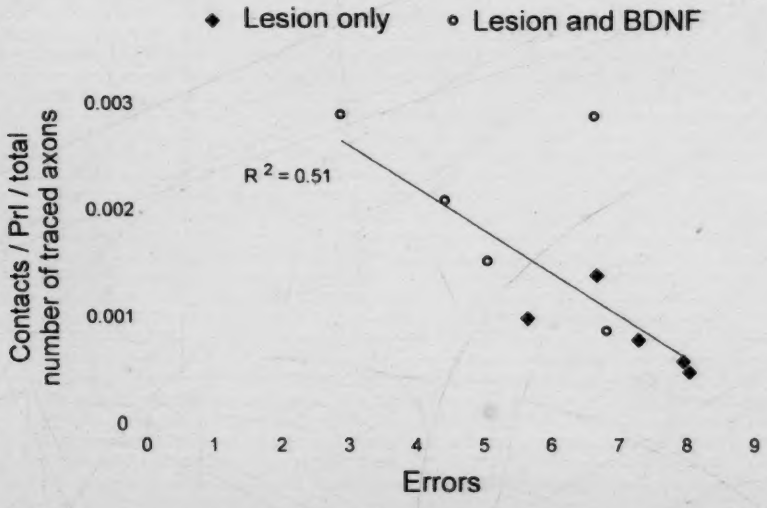

Figure 2.8. A correlation of the number of contacts (normalized to the total number of traced axons and the number of PrI as shown in figure 6B) between CST and PrI and the performance on the horizontal ladder is statistically significant. Single points symbolize the average values for single animals.

Table 2.1

Treatment group	# of traced axons	mm analyzed	# of collaterals	# PrI
no lesion no treatment	766 ± 299	150 ± 7	154 ± 69	398 ± 192
lesioned + BDNF to cortex	791 ± 186	121 ± 10	256 ± 125	419 ± 207
lesioned + vehicle to cortex	975 ± 167	121 ± 11	130 ± 17	70 ± 21
lesioned + NT-3 at C3	1158 ± 160	151 ± 14	127± 95	278 ± 73
lesioned + vehicle at C3	1607 ± 364	129 ± 18	95 ± 12	158 ± 71

Table 2.1. Averages of originally counted values (i.e., total number of traced axons, length of tissue analyzed, total number of collaterals, and total number of PrI) that were used to normalize the data.

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CHAPTER 3

REACHING TRAINING PROMOTES PLASTICITY IN PARALLEL WITH FUNCTIONAL RECOVERY FOLLOWING CERVICAL SPINAL CORD INJURY IN RATS

3.1 Introduction

Nearly 50% of all human spinal cord injuries (SCI) occur at the cervical level resulting foremost in impairments in upper limb function (Anderson et al., 2005). In a recent survey of patients living with SCI, regaining hand function was listed as the central concern for quadriplegics (Anderson, 2004). Even small gains in arm/hand function translate into considerable improvements in independence (Popovic et al., 2006) and subsequently, quality of life.

Recovery following SCI is limited due to several factors including the glial scar formed around the injury and the presence of inhibitory molecules on damaged oligodendrocytes (Yiu & He, 2006). Although long distance regeneration of injured axons does not occur naturally, anatomical rearrangements may spontaneously occur in the injured CNS. Injury-induced sprouting of corticospinal tract (CST) fibers has been found above (Fouad et al., 2001) and below (Weidner et al., 2001) a spinal lesion. Additionally, sprouting of reticulospinal tract (RtST) axons below a spinal lesion (Ballermann et al., 2006a) has also been shown. A recent study has also shown that the sprouts found after a lesion may make meaningful connections onto interneurons, forming new intra-spinal connections within the spinal cord (Bareyre et al., 2004). These new connections are thought to possibly contribute to the spontaneous functional recovery seen following SCI, and, in a later study, were shown to correlate with behavioural improvements after SCI (Vavrek et al., 2006).

One treatment which may promote functional recovery following CNS injury (possibly by enhancing injury-induced plasticity) is increased physical activity. Various methods of increasing physical activity exist for both animal models and human cases of

CNS injury. CIMT (to minimize the effect of learned non-use of the injured limb) following stroke in rodents (DeBow et al., 2001) and humans (Ro et al., 2006), and rehabilitation training (without restraint of the "unimpaired" limb) following SCI in humans (Beekhuizen et al., 2005) have been shown to promote functional recovery. Following SCI affecting the lower limbs in patients, treadmill training has been used extensively (Dobkin et al., 2006). The basis of treadmill training is rooted in findings of recovery of hind-limb function after treadmill training in spinalized cats (Lovely et al., 1986). Although the mechanisms of recovery following training are not fully known, it is likely due to disinhibition of spinal networks (de Leon et al., 1999), plasticity (sprouting above (Fouad et al., 2001) and below (Ballermann et al., 2006a; Weidner et al., 2001) the lesion) which may ultimately lead to the formation of compensatory movement strategies (Ballermann et al., 2006b). Although increasing physical activity has been shown to promote functional recovery in both humans and animal models, there may be some negative effects of exercise. Several reports have shown that there is a sensitive period following brain injury in which implementation of increased physical activity results in impairment (increased lesion size and decreased functional recovery; DeBow et al., 2004; Humm et al., 1998) but this may not be the case following SCI (Norrie et al., 2005). Furthermore, the recovery found following training in cats and in humans appears to be task specific, where recovery of function in one task does not imply recovery in the other. Spinalized cats trained to stand are unable to walk on a treadmill as fast as rats trained to walk (Edgerton et al., 1997; de Leon et al., 1999). In humans, training of forward walking on a treadmill until patients were able to walk with very little assistance did not translate

into backwards walking once the direction of the treadmill belt was reversed (Grasso et al., 2004).

The beneficial effects of physical activity seen in animal models and humans may be due to several upregulated (and downregulated) factors within the CNS. Increased expression of neurotrophins (BDNF, NT-3, NT-4; Gomez-Pinilla et al., 2001, 2002; Skup et al., 2002; Ying et al., 2003, 2005), neurotrophin receptors (TrkB & C; Skup et al., 2000) and growth associated protein (GAP)-43 (Azcurra et al., 2003) are all found in the CNS of animals following increased physical activity. Furthermore, a downregulation of NgR (myelin based inhibitors receptor; Josephson et al., 2003) is also seen.

Considering the benefit of increased physical activity following stroke and SCI affecting the lower limbs, it is surprising that very few laboratories have tested rehabilitation training following cervical SCI in animal models or human patients. In the current study, we used a well-described behavioural task (single pellet reaching) as a means of providing specific training to adult rats following a cervical spinal cord injury. We hypothesized that specific training would promote functional recovery following cervical SCI, possibly via sprouting of a descending tract (the CST).

3.2 Methods

3.2.1 Animals

Fifty female Lewis rats were group-housed (4-6 per cage) and kept on a 12h:12h light/dark cycle. To minimize grasping motions of the forelimbs in the cage, rats were fed rodent chow mash (made up of rodent chow and water). All rats were given *ad libitum* access to water. Adult rats weighed at least 170g at the time of SCI. In total, 3 rats were

excluded from the study due to a large lesion and, subsequently, an inability to perform the grasping task (see below)). This study was approved by the local animal welfare committee and complies with the guidelines of the Canadian Council for Animal Care.

3.2.2 Single pellet reaching task

The chamber (Figure 3.1) used was built based on previous reports (Wishaw et al., 1993) of clear Plexiglas with the following dimensions: 45cm in height, 12.5cm in width (inside width), 38.5cm in length. The floor of the chamber was made of Plexiglas for the back half and metal grating for the front half (to ensure that a dropped pellet would remain unretrievable). In addition, the entire chamber was raised 3cm from the ground so that dropped pellets could not be retrieved. The shelf where the pellets were deposited measured 5cm×5cm and rested at 3cm from the base surface of the chamber. This distance was far enough that the rat could not retrieve the pellet with its tongue, but could grasp for it with its preferred forelimb. A small opening (1cm wide) allowed for the rat's paw to reach through for a pellet placed in a small indentation 2cm from the inside wall of the chamber aligned to either the left or right edge of the opening depending on the rat's handedness.

Figure 3.2 shows a timeline of the current study. All rats were trained for 3 weeks on the single pellet task before being subjected to SCI (see below). Animals were given one single training session per day, 6 days per week. During training sessions, rats were given access to 45mg chocolate flavoured sucrose pellets (Research Diets, New Brunswick, NJ, USA) for 10 minutes. A trial was scored as a success once the animal successfully retrieved a pellet from its well and ate it (the pellet remained available to the

rat until it was either retrieved (success) or knocked off the shelf by the rat or pulled into the chamber but dropped through the metal grating (failure)).

Once baseline scores for all the animals reached a plateau (~65% success rate), the rats were recorded for 10 minutes using a digital video camera (JVC, 30 frames/s; shutter speed: 1/500s), and for 2-3 further grasps with a high speed video camera (IPX-VGA 210 IMPERX Inc, Boca Raton, FL, USA) captured with StreamPix (NorPix Inc, Montreal, PQ, 200 frames/s).

3.2.3 Qualitative analysis of skilled reaching

For qualitative analysis of single pellet reaching, a reach was broken down into 7 components (Metz and Whishaw, 2000). Briefly, each of the 7 components contained the following characteristics. (1) *Advance*: the forelimb moves forward through the slot and moves toward the pellet. As the forelimb advances the elbow continues to move in. (2) *Digits open*: the digits open and partially pronates above the pellet. (3) *Pronation*: the elbow moves out, the palm is fully pronated over the pellet and the palm moves down in an arpeggio movement (1 digit at a time). (4) *Grasp*: the digits close around the pellet. (5) *Supination I*: the elbow moves in as the palm is withdrawn. As the palm is being withdrawn the palm turns 90°. (6) *Supination II*: the palm is withdrawn from the slot and the palm is again supinated so that the palm faces the rat's mouth. (7) *Release*: the rat sits back and places the food in its mouth. The rat also will raise its other paw to assist the reaching paw with eating.

Each of the components was scored on a 3-point scale. A score of 1 was given if the movement was present. A 0.5 given if the movement was present but abnormal, and a score of 0 was given if the movement was absent.

Scores for each rat were averaged from 3 individual reaches recorded on a digital video camera (see above) from their baseline testing, and final testing (see below). Final scores were expressed as a percentage of the baseline score.

3.2.4 Horizontal Ladder Walking Task

Prior to being subjected to SCI (see below), rats were familiarized with the horizontal ladder walking task. The horizontal ladder is 1m long and fitted with irregularly spaced metal rungs (~2cm apart). The entire apparatus is raised 30 cm from the ground. To familiarize the rats, each rat crossed the ladder 3 times on 2 separate days.

3.2.5 Spinal Cord Injury

During baseline single pellet task training, the preferred paw for each rat was determined. SCI lesions were performed to the spinal cord ipsilateral to this paw. We wanted to examine 3 separate unilateral lesion paradigms: CST, RST and combined CST/RST. Animals were randomly assigned to each lesion paradigm and were further assigned to control or treatment conditions 3 days later (see below).

To perform the lesions, rats were anesthetized using gas anesthesia (4% isofluorane for induction, 1-1.5% for maintenance) a partial laminectomy of the C3 cervical vertebrae was performed. The spinal cords were then lesioned using a microblade. The muscle and skin overlaying the injury site was sewn using silk suture.

The rats were then given 0.03mg/kg buprenorphine and 4ml of saline immediately after the surgery (and were given buprenorphine and saline for up to 2 days following SCI if they showed signs of pain or dehydration) and placed on a heating pad until awake. At 3 days following injury (when the rats were separated into their groups) forelimb deficits ranged from digit paralysis to complete forelimb paralysis up to the elbow. Animals displaying deficits more severe that forelimb paralysis to the elbow (e.g. forelimb paralysis up to the shoulder) were euthanized (n=3).

3.2.6 Post-injury training

Three days following SCI rats (after being separated into their lesion types) were paired according to their deficits. The pairs of animals were then separated into two groups (trained and untrained). This ensured that both groups had similarly injured animals. Starting on day 4 following injury, animals were trained for 6 weeks (6 days/week, 10 minutes/day). Untrained animals were placed in the grasping chamber but were not given access to the single pellet shelf.

The majority of the trained rats continued to grasp with their preferred (injured) forelimb, but if they attempted to use their other (uninjured) forelimb, gauze was taped to the forelimb to prevent it from being able to reach through the opening. In most cases, this was only necessary once or twice, until the animal learned that it could not grasp for pellets using that paw.

On their first training session, most rats in the training group attempted to grasp for pellets, but were unsuccessful. Rats with combined lesions (to the CST and RST) were able to lift their limbs up high enough to reach through the opening, but were

sometimes unable to guide their limb through the opening. In these cases, a pellet was held with forceps close to the opening to the shelf until the rat would use its forelimb to remove the pellet from between the forceps. Over time, the forceps were moved further and further back (towards the well) until the forceps were no longer needed and the rat could reach through the opening and retrieve the pellet from the well.

3.2.7 Final testing

Following six weeks of training, all rats were tested on the single pellet task and the horizontal ladder walking task. Rats were recorded performing the single pellet task using the same method as with baseline testing. Rats were tested on the single pellet task over 3 days to ensure that the untrained rats had sufficient time to "relearn" the task. On the final testing day, rats were video-recorded (digital video camera as above) crossing the horizontal ladder 3 times.

Detailed analysis of grasping style was performed from video recordings (see above). Horizontal ladder walking error rates were calculated by adding all slips (noted once the animal placed the forelimb on a rung, but the forelimb then slipped off the rung before making a new step) and falls (noted once the animal placed its paw in between two rungs and did not make contact with any rungs before making a new step) made by a paw, dividing that number with the number of steps taken by that paw, and then multiplying by 10 steps (i.e. all errors were expressed as the number of slips and falls per 10 steps).

3.2.8 Electrophysiology

Electrophysiological assessments (cortical map, and motor evoked potentials (MEPs) in the forelimbs) were conducted on all animals following their final behavioural testing. Animals were anesthetized and placed into a stereotaxic frame. The skin above the skull was cut and two rectangular openings were drilled into the skull overlaying the forelimb motor area. The coordinates for the openings were: 1mm lateral to 4mm lateral to bregma and extended to 3mm rostral to bregma. A tungsten microelectrode (FHC Inc., Bowdoin, ME, USA) was used to deliver a train of cathodal pulses (n=30, 0.25ms, 330Hz) to the cortex. Four electrodes (2 per forelimb) with exposed tips (3 stranded Teflon-coated wire, A-M Systems, Carlsborg, WA, USA) were inserted into both forelimbs (group of extensor muscles on the dorsal surface of each forelimb) and used to record MEPs. The EMG signals were amplified (Cyber-Amp 380, Axon Instruments, Forster City, CA, USA), digitized (5kHz, Digidata 1322A, Axon Instruments, Forster City, CA, USA) and filtered (30-300Hz) using the Digi-Data interface. During stimulation, isofluorane levels were maintained as low as possible (~1%). On average, 15 spots/opening (30 spots/animal) were stimulated. The microelectrode (attached to the stereotaxic frame) was always inserted at the center of the opening first (approximately 1.5mm rostral and 2.5mm lateral to bregma) to a depth of 1.5mm. At each spot, the electrode was lowered up to 2.0mm into the cortex until the maximal response (from visual observation) at 200μA was reached. To determine the threshold to elicit the same response, the stimulus intensity was lowered 10μA at a time until the response no longer occurred. The response type (e.g. wrist, shoulder, whisker movement) and the threshold to elicit the response were noted for each spot stimulated. The cortex ipsilateral to the

injury (i.e. the cortex connecting to the "uninjured" forelimb) was always stimulated first (as control).

In most cases, an incomplete map of the CST was collected from the rats. This was due to several reasons. Firstly, the blood vessels overlaying the cortex prevented the stimulation of all cortical areas. Once a blood vessel or one of its smaller (difficult to detect) branches was cut by the electrode, we were usually unable to obtain any more MEPs from the rat. Furthermore, this was not a terminal experiment, and tracing (see below) of the CST was performed immediately following stimulation. Since each surgery took close to 3 hours to complete, stimulations were terminated once rats showed signs of irregular breathing.

Cortical Map: To obtain group cortical maps for trained and untrained rats in the CST/RST and RST only groups, we quantified the occurrence rate of forelimb movements at each stimulated point in the forelimb cortical area. Forelimb movements were only included in the maps if they were elicited from stimulation of the contralateral cortex. For each point in the cortical area, the number of animals in which a forelimb (digit, wrist and/or elbow) movement was elicited was taken over the number of times that spot was stimulated in each group. For example, if only 1 animal in a group responded to cortical stimulation when 5 of the animals in the group were stimulated at that point, the occurrence rate would be 0.2. To attempt to eliminate outliers, only spots with a minimum of 2 stimulations (i.e. only when two animals per group had been stimulated at that point) were included in the map. If a spot had been stimulated 1 (or 0) times, then the average occurrence rate of the spots around it was taken as the occurrence

rate for that spot. Finally, a 4-colour colour code was given to the map in order to visualize areas with higher forelimb-movement occurrences.

3.2.9 Tracing

At the end of the electrophysiological experiments, the CST emanating from the forelimb area of the cortex of all rats was traced with 10% biotinylated dextran amine (BDA, 10,000 MW, Molecular Probes, Invitrogen Corporation, Burlington, ON). A 1µl Hamilton syringe was inserted into the side of the cortex contralateral to the injury (i.e. to trace the injured CST) at 1.5mm lateral to and 1.5mm rostral to bregma. Once lowered to 1.5mm deep, 0.8µl of BDA was injected over a period of 5 minutes. The skin overlaying the skull was sutured using silk and the animal was given 0.03mg/kg buprenorphine. Animals were then placed on a heating pad until awake.

3.2.10 Perfusions & histology

Two weeks following tracer injection, animals were euthanized with an overdose of pentobarbital (1.6ml/kg) and then transcardially perfused with saline and fixed with 4% formalin in 0.1M phosphate buffer, with 5% sucrose added. Brains and spinal cords were extracted and post-fixed in 4% formalin (with 5% sucrose) overnight and then cryoprotected with 30% sucrose over 3 days.

The spinal tissue from each animal was divided into 2 blocks; A C1 segment (mounted for cross-sections and used to count the number of fibers traced) and the remainder of the cervical segments (mounted for horizontal sectioning and used to both verify lesion size and count the number of collaterals rostral and caudal to the lesion).

Both blocks were embedded in Tissue Tek (Sakura Finetek USA Inc., CA, USA) and frozen in 2-methyl-butane over dry ice (~-60°C). All tissue was sectioned at 25μm in a cryostat at -20°C. Sections were mounted onto slides and were stored at -20°C until ready for immunohistochemistry.

Sections were stained for BDA. All slides were reheated at 37°C for 1 hour to adhere sections to the slides and then rehydrated with two 10 minute washes of TBS followed by two 45 minute washes in TBS-TX. Slides were incubated overnight (at 4°C) in avidin-biotin complex (ABC) solution (Vector Laboratories, Burlingame, CA). The following day, following a rinse (two 10 minute washes in TBS) slides were processed with DAB (Vector Laboratories) according to the instructions of the manufacturer. Briefly, DAB solution was placed over the slides for a maximum of 5 minutes and then placed in distilled water to halt the reaction. Slides were then washed (two 10 minute washes in TBS) and dehydrated (2 minutes in each of the following: 50%, 75% and 100% alcohol and then cleared with two separate xylene washes). Sections were then coverslipped with Permount.

Injury site: The injury site was reconstructed by analyzing every fourth horizontal section using light microscopy (10x magnification). The maximal extent of the injury was determined using landmarks (relative amount grey/white matter, emergence of the CST, central canal) and mapped onto a schematic of the cervical spinal cord. The lesion size was calculated from the schematic using Scion Image (NIH).

C1 sections: Due to variability in the total number of CST fibers traced in each animal, C1 sections were used to normalize the number of traced fibers. Photographs of a C1 section from each animal were taken using a camera mounted onto a Leica microscope (40x magnification). The total number of labeled CST fibers was counted for each animal.

Collaterals: Collaterals were counted in a similar manner as previously described (Chapter 2). Briefly, each time a CST fiber crossed into the grey matter for a distance of at least 2μm was counted. For animals with incomplete CST lesions, the number of collaterals extending into the grey matter was counted below (rostral) and above (caudal) to the lesion. The distance of the CST analysed per each section was also noted. The final number of collaterals was expressed as # of collaterals/fiber (counted on the C1 section)/mm (distance analysed).

3.2.11 Statistics

Statistical analysis was performed using a non-parametric student's t-test (GraphPad Prism, San Diego, CA, USA) unless otherwise noted within a Results section. If variances between two groups were significantly ($p<0.05$) different, a Welch's correction was performed. When comparing success rates in untrained animals during their final testing days, a paired non-parametric t-test and a one-way ANOVA was used. When quantifying cortical maps, a paired non-parametric t-test was used. All data are presented as means ± standard error.

3.3 RESULTS

3.3.1 Lesion size between trained and untrained rats is comparable

Before being randomly assigned to either training or no training groups, animals were paired according to their SCI-induced deficits to ensure that lesion sizes would be similar in each group. Rats in the training group were then retrained on the single pellet reaching task the next day, and were trained for 6 weeks (6 days/week, 10 minutes/day).

Following brain injury, there appears to be a sensitive period following injury in which increasing physical activity increases lesion volume (DeBow et al., 2004), but the presence of such a period following SCI has not yet been determined. In the current study, we did not observe an exacerbation of lesion size (expressed as the percentage of lesioned tissue over the entire spinal cord cross-section size) following 6 weeks of training when we included all lesioned rats (data not shown; untrained: 25.3%±1.7% n=23; 25.2%±2.4% n=24).

Figure 3.3 shows schematics of lesion sizes (A) and quantification of lesion sizes in all three subgroups (B). Some of the rats in each lesion group had to be excluded from further analysis due to incomplete lesions of descending tracts. Animals were included in the CST or RST group if there was no more than 5% sparing of the respective tract and no injury of the other tract. Animals in the CST/RST group were included in this group only if they had 5% (or less) sparing of the tracts. All further analyses were completed with the following sample sizes per group: CST injury (trained: n=2, untrained: n=6), RST injury (trained: n=5, untrained: n=6) and CST/RST injury (trained: n=8, untrained: n=6). Trained and untrained animals did not significantly vary in lesion size in any of the

subgroups (CST/RST: 29.1±2.5% vs. 35.0±3.0; RST: 10.9±1.5% vs. 22.8±3.7%; CST: 27.7±10.7% vs. 21.6±2.6% respectively).

3.3.2 Training increases single pellet reaching task success rate in CST/RST injured rats

Final testing (including testing of the single pellet reaching task) was completed over 3 days, to attempt to control for variability in the performance of each rat. The best score achieved out of their 3 testing days was used for comparison to their best baseline score achieved during pre-injury (baseline) training for each rat. The final success rate was taken as a percentage of the baseline score up to a maximum of 100% (100% success rate meaning that the rat achieved the same success rate after injury as during baseline testing). In untrained rats, success rates on the reaching task increased slightly from day 1 to day 2 (data not shown; $p=0.2626$) but leveled off by the third testing day. Overall, there were no changes in success rate over the 3 testing days in untrained animals (data not shown; $p>0.2277$).

Figure 3.4 illustrates success rates in all three of the lesion subgroups between trained and untrained rats. Success rate comparisons between trained and untrained rats yielded a significant difference in only the CST/RST lesioned group and not the CST group or the RST group. In the CST/RST group, trained rats performed significantly better on the reaching task compared to their untrained counterparts (untrained: 33.5±6.2%, trained: 60.9±4.5%; $p=0.0047$). There was no significant difference in success rates of trained and untrained rats in the RST only or CST only.

3.3.3 Training does not alter grasping style in CST/RST injured rats

Detailed analysis of grasping style was meant to describe the different strategies animals adopted following injury and to determine whether these strategies were further altered by training. Detailed video analysis was only completed for animals in the CST/RST and RST groups because a low group size in the CST trained group (n=2) is not sufficient to yield useful data with this detailed analysis. Seven different phases in the grasp were analysed and quantified (see methods).

In the lesion group where training promoted functional recovery (CST/RST group), training did not significantly alter the grasping technique that animals used at any phase of the grasp when compared to their untrained counterparts (Figure 3.5A). In all CST/RST injured animals (trained and untrained), however, phase 3 of the grasp (where the palm is placed down upon the pellet and the digits close around it in an arpeggio motion) is almost completely absent, in keeping with previous reports (Whishaw et al., 1998) showing the arpeggio movements of the digits to be absent in animals with a CST and RST lesion.

Training did alter two phases of the grasp in the RST group (phase 4 (grasp: where the digits close around the pellet); trained: 0.65±0.1, untrained: 0.97±0.02; p=0.0382 (*t*-test with Welch's correction) and phase 6 (supination II: where the pellet is removed from the shelf and placed near the rat's mouth); trained: 0.52±0.07 untrained: 0.79±0.09; p=0.0472; Figure 3.5B). These significant changes in grasping style did not translate into a difference in success rate in the RST injured trained animals and appears to be behaviourally unimportant.

3.3.4 Increased occurrence of motor evoked potentials in trained CST/RST injured rats

Cortical maps are presented as the ratio of occurrence of forelimb movements. Briefly, for each of the cortical areas stimulated, the number of animals in which a forelimb movement was elicited was taken over the total number of animals stimulated at that point, giving a ratio of forelimb movements for each group and treatment condition.

In Figure 3.5A, the maps collected from the cortex contralateral to the injury (controlling the forelimb with a CST injury) are presented. These maps show only the occurrence of movements of the "injured" forelimb. As can be seen, changes occur in every group compared to control rats (shown at the center of the figure).

Cortical map quantifications were done by using the divisions from the colour code used in Figure 3.6A, and are presented in Figure 3.6B. The occurrence of forelimb movements increased compared to controls in all lesion/treatment paradigms except for CST/RST untrained rats. The largest increases were seen in the RST injured groups, with RST untrained ($p<0.0001$) and trained ($p=0.0025$) having significant increases compared to control uninjured rats. Training in the RST injured group actually significantly decreased the occurrence of forelimb movements compared to untrained RST injured rats ($p=0.0479$).

CST/RST untrained rats did not have an increase in the number of MEPs found after injury alone ($p=0.10$, compared to uninjured rats). In parallel with the enhanced recovery seen on the single pellet reaching task, CST/RST trained rats, showed increases in the occurrence of MEPs compared to both control uninjured rats ($p=0.0295$) and untrained CST/RST injured rats ($p=0.0413$).

A different pattern is seen in the ipsilateral (to injury) cortex (i.e. controlling the uninjured forelimb; Figure 3.6). All injury/training groups show an increase in the occurrence of forelimb movements (except for CST/RST untrained; $p=0.1465$), when compared to control uninjured rats. CST/RST trained rats ($p=0.0134$), RST untrained rats ($p=0.0067$) and RST trained rats ($p=0.0448$) all show increases in forelimb movements, but neither of the injury subgroups show changes when compared between them.

Due to a low sample size in the CST trained subgroup ($n=2$), cortical maps from this group (and the untrained CST group) are not shown in Figures 3.6 or 3.7. As described in the methods, the blood vessels overlaying the cortex prevented stimulation of all cortical spots. In all others groups, the higher sample size allowed for group maps to be made because any one cortical area was likely stimulated in at least 2 of the animals. With a lower sample size, this becomes less likely, and a useful map could not be made.

3.3.5 Training does not alter thresholds or delays in injured rats

The minimum threshold to evoke a movement of the injured forelimb following stimulation of the contralateral cortex was not significantly different in any group following training (see Figure 3.8). Although thresholds in most injured rats were increased compared to uninjured control rats, this did not reach statistical significance.

Delay to evoke muscle activation (from the onset of the stimulus) was calculated when EMGs were collected from the animal (see Figure 3.9A for an averaged motor evoked potential (4 traces) collected). There was no significant change in the delay to evoke a motor response in the forelimb in any group following training (see Figure 3.9B).

3.3.6 *Training increases the number of CST collaterals in cervical enlargement of CST/RST injured rats*

Figure 3.10A shows a sample horizontal section of the spinal cord from an injured rat, with CST collaterals illustrated with an arrow. Figure 3.10B shows the quantification of the collaterals in uninjured and injured (trained and untrained) rats.

The number of collaterals emerging above or below the injured CST was calculated as described previously (Vavrek et al., 2006). The number of CST collaterals rostral to the lesion was used to compare between groups and to control (uninjured) rat values.

In parallel with enhanced functional recovery on the single pellet reaching task, and increased occurrence of MEPs, CST/RST trained rats had an increased number of CST collaterals compared to control (uninjured) rats ($p=0.0467$). CST/RST untrained rats did not differ from control rats in terms of collateral sprouting rostral to the lesion ($p=0.1239$). In RST injured rats, the number of CST collaterals (a completely uninjured tract in these animals) was increased compared to uninjured rats ($p=0.0347$). RST trained rats, however, did not have an increased number of CST collaterals in comparison to control uninjured rats ($p=0.1658$). CST trained ($p-0.6429$) and untrained ($p=0.1197$) rats did not have a significant increase in the number of CST collaterals compared to control uninjured rats. No significant difference in the number of collaterals rostral or caudal to the injury was found between trained and untrained animals in any group.

3.3.7 Reaching training interferes with ladder walking

Figure 3.11 shows the performance of all 3 subgroups on the horizontal ladder task. The horizontal ladder walking error rate was expressed as the number of slips and falls made by a paw per 10 steps. The error rate for the injured forelimb was used for statistical analysis as there was no change in the number of errors made with the "uninjured" forelimb in any of the groups with or without training. The majority of lesioned rats showed significant impairment on the task compared to unlesioned animals. Control (unlesioned) animals seldom make errors with their forelimbs on the horizontal ladder walking task, but nearly all lesioned animals in each of the subgroups made errors with their preferred (injured) paw. Error rates between trained and untrained rats varied in two of the subgroups. In the CST/RST group (the same group which showed significant improvement on the grasping task following training) there was a significant decline in the horizontal ladder walking task (trained: 1.91±0.4, untrained: 0.70±0.3; p=0.0281). Trained rats in the CST only group also made significantly more errors on the horizontal ladder compared to their untrained counterparts (trained: 3.85±1.5, untrained: 1.24±0.4; p=0.0483). There was no significant difference in error rates between trained rats in the CST injured and CST/RST injured rats. Rats in the RST only group make the same number of errors on the gridwalk whether or not they are trained (trained: 1.03±0.3, untrained: 0.95±0.4; p=0.8667).

3.4 Discussion

In this study, we showed that specific (grasp) training can promote functional recovery following dorsolateral quadrant cervical SCI in rats. This is the first time that

training of a volitional task controlled by descending tracts in rats (rather than a pattern generating network (as in spinalized cats)) has promoted functional recovery following SCI. Functional recovery was promoted only in rats with a combined CST *and* RST injury, whereas rats subjected to a CST or a RST injury did not have enhanced recovery on the single pellet reaching task. In the following sections, the results from the three lesion subgroups will be considered separately.

3.4.1 CST/RST injured rats

Functional recovery in CST/RST injured trained rats: Six weeks following combined injury to the CST and RST, rats show a decline in the performance of the single pellet reaching task, down to approximately 30% of baseline. Six weeks of near-daily training (6 days/week, 10 minutes/day) on the single pellet reaching task is sufficient to promote the recovery of this task to over 60% of baseline, over double that of untrained animals. The level of recovery in the current study is well over that of a study using a pharmacological agent (NT-3) in a similar lesion paradigm (Lynskey et al., 2006).

Mechanisms of recovery in CST/RST trained rats: To determine how CST/RST trained rats were able to recover the ability to perform the single pellet reaching task, we analysed behavioural, electrophysiological and histological data from these animals.

First, detailed grasping style analysis allowed us to determine that training did not alter the grasping style used by these animals to retrieve pellets. This means that, qualitatively speaking, CST/RST injured rats are as able to grasp for pellets normally with or without training. One striking difference seen in CST/RST injured rats is the near

disappearance of the third phase of the grasp. Pronation (the third phase) is the phase in which rats place their paws down on the pellet with an arpeggio movement of the digits. The finding that this phase is abolished with CST/RST lesions is in keeping with previous reports (Whishaw et al., 1998) that show the arpeggio movements of the digits to be absent in animals with a combined CST and RST lesion.

Second, cortical maps show an increased occurrence of MEPs in the forelimb area contralateral to the injury (i.e. the cortex projecting to the injured forelimb) in CST/RST trained rats in comparison to untrained CST/RST injured rats and also control uninjured rats. On the other side of the cortex (i.e. the side projecting to the uninjured forelimb), the occurrence of MEPs was again increased in trained rats compared to uninjured rats, but not compared to untrained rats. This may be due to the fact that following a lesion, rats in the single pellet reaching task have to lean onto their uninjured limb more than before the injury in order to support themselves. Additionally, rats will attempt to use the uninjured limb to aid the injured limb in retrieving pellets (e.g. supporting the injured limb with their uninjured limb).

Forelimb movements (of the injured forelimb) were elicited in nearly all CST/RST injured rats. There are several explanations for this result. Although the majority of the rats in the CST/RST group had a completely lesioned CST, animals were still included in this group if they had no more than 5% sparing of the tract. Furthermore, lesioning the dorsolateral quadrant of the spinal cord will completely spare the ventral CST, which accounts for approximately 5% of all CST fibers. Importantly, the majority of the ventral fibers terminate at the cervical level, suggesting that they affect forelimb function most strongly (Brosamle & Schwab, 1997). Additionally, several changes within

the cortex may also account for the cortical map changes seen. Although it may not be verified from the data of the current study (since the cortices were not analysed), axonal sprouting/growth of new arbors, circuit rearrangement and activity-dependent increases in synaptic efficacy (Donoghue, 1995) may explain the cortical map changes seen in the CST/RST trained animals.

Another mechanism which may explain cortical map changes is threshold reductions. A reduced threshold to elicit an MEP would likely result in an increased number of points responding to stimulation. A reduced threshold was not seen in the current study when compared between trained and untrained rats or when compared to uninjured control rats. It is possible that the anesthesia used in the current study prevented us from seeing small reductions in thresholds (due to a very low excitability), but nevertheless no threshold changes were seen.

One last electrophysiological test used in the current study was the delay to elicit an MEP. As with the threshold data, we did not observe any change in the delay to elicit a forelimb movement when we compared trained and untrained rats. We do not have any control (uninjured rats) data to compare our delay results to, and so there may have been a difference between injured rats and uninjured rats.

The last mechanism we hypothesized could be related to the recovery of function and the cortical map changes seen, was collateral sprouting of the injured CST. We found a significant increase in the number of CST collaterals emanating from the injured CST of trained CST/RST injured rats to that of control uninjured rats. These increases were found at a level above (rostral to) the injury in CST/RST injured rats. It is possible that these collaterals connected onto interneurons within the cervical enlargement, or,

alternatively may have connected directly onto motorneurons forming new connections within the injured spinal cord. It is surprising that CST/RST injured untrained rats did not have an increased number of collaterals just from injury in comparison to control uninjured rats. In previous reports showing injury-induced sprouting of the CST, the SCI is at the thoracic level (Bareyre et al., 2004; Fouad et al., 2001), and the sprouting is found at the cervical enlargement. In the current study, the SCI was at the cervical enlargement, and the sprouting was found directly above (C2 and C3) this lesion. It is possible that increased sprouting can only be found in the cervical enlargement if the injury is further caudal (in the thoracic spinal cord). In fact, in a previous study, no increase was found in the number of CST collaterals in the thoracic spinal cord following a thoracic SCI (Chapter 2). Furthermore, the size of the injury may have played a part in the lack of axonal sprouting due to the injury in the no-training group. Judging from the results of previous studies, it appears that axonal sprouting in the cervical enlargement may be increased following a lesion, but only if the lesion is relatively small, and leaves sufficient descending targets for the sprouts to connect onto. For example, a lesion of the dorsal columns (thoracically) enhanced the sprouting of the injured CST in the cervical enlargement (Bareyre et al., 2004; Fouad et al., 2001) whereas a dorsal overhemisection (lesioning approximately 75% of the spinal cord) did not (Vavrek et al., 2006).

It appears as though functional recovery was obtained in CST/RST injured rats following 6 weeks of training in parallel with cortical map changes and sprouting of the injured CST. Interestingly, however, enhancement in the performance of the grasping task, following training did not translate into enhanced performance on another related

task requiring fine motor control, but actually caused a deficit in the performance of this task.

Training interferes with horizontal ladder walking in CST/RST injured rats: Six weeks of training on the single pellet reaching task was sufficient to enhance the performance of this task in CST/RST injured rats over that of untrained rats. The horizontal ladder walking task, like the single pellet reaching task, is a task of fine motor control requiring descending input from the CST and RST. To determine if enhanced performance on the single pellet task would transfer to the ladder walking task, we counted the number of errors rats made with their injured (and preferred) forelimb. Normally, uninjured rats will make very few (nearly zero) errors with their forelimbs on the horizontal ladder walking task. Following combined injury to the CST and RST, rats made approximately 1 error per crossing of the ladder. CST/RST trained rats, however, made significantly more errors with their injured forelimb, doubling the number of errors to approximately 2 errors per crossing of the ladder.

Proposed hypothesis explaining results in CST/RST injured rats: Single pellet reaching task training in CST/RST injured rats significantly enhanced performance of that task while significantly decreasing performance of a related task (the horizontal ladder walking task). This has been seen previously, to various degrees, following treadmill training in spinalized cats and partial SCI patients. Spinalized cats trained to stand (i.e. support their weight) walk significantly slower on a treadmill compared to spinalized cats trained to walk on a treadmill (de Leon et al., 1999) In human SCI patients, treadmill

training is able to promote the recovery of forwards walking, but once the treadmill belt direction is reversed (i.e. for backwards walking) all SCI patients were unable to walk backwards (Grasso et al., 2004). These results are still somewhat different than ours. First of all, and most importantly, they are most likely not due to changes/reorganizations in descending input, since the subjects were either completely spinalized (the cats) or had severe, near-complete lesions (human patients). The results are most likely due to changes within the CPG. For example, treadmill training exerts its effects, at least in part, by decreasing inhibition within the spinal cord (de Leon et al., 1999). Secondly, these results show that spinal cord injured cats or humans trained to perform one task cannot transfer their enhanced performance of that task onto other tasks (in the case of human SCI patients) or cannot perform an untrained task as well as separate animals trained to do this task (in the case of spinalized cats). In our study, we observed an increased performance of a trained task (in trained animals) and a decreased performance of an untrained task (in the same trained animals). Since grasping is orchestrated within motor areas of the cortex, and most likely does not require a CPG, these results must be due to changes within descending tracts.

There are several possible mechanisms which may explain the results seen in our experiment. The first and most likely situation is that single pellet reaching training following SCI actually exacerbated the injury size. Following brain injury in rats, excessive use of the injured forelimb immediately following injury has been shown to increase injury size and decrease functional recovery. If that were the case in our rats, the 6 weeks of single pellet training may have enabled the rats enough practice in that task which would serve to mask the fact that they were actually more severely injured.

We compared lesion sizes in the CST/RST injured trained and untrained animals and found no significant change in lesion size. Furthermore, we compared lesion sizes in all trained and untrained rats and also found no significant change in lesion size (data not shown). It is unlikely that lesion size caused the results seen in this injury group.

Another possibility for the results is the competition for neural substrates within the premotor area following training. If we assume that although the single pellet reaching task and the horizontal ladder task are similar, but still require separate input from the premotor area, we may be able to see how specific training would reduce performance of other related tasks. Once rats are subjected to training on the single pellet reaching task, the connections between the premotor area responsible for grasping, through the brain, spinal cord and eventually motor neurons and muscles may be reinforced, whereas connections from the premotor area responsible for ladder walking (to motor neurons) would not be used, and would be weakened. Once those connections would be weakened and possibly even lost, they could be then overtaken by the grasping area, resulting in an expansion of the cortical area responsible for grasping and a reduction in the cortical area responsible for ladder walking. This theory cannot be fully determined with the data from this current report, but further studies may aid in strengthening or refuting the idea.

3.4.2 RST injured rats

Although rats with an RST injury did not demonstrate an enhancement of functional recovery following training of the single pellet reaching task, nor a change in

performance on the horizontal ladder walking task, there were several changes seen following training in this group, and also following injury alone.

Training alters the grasping style in RST injured rats: Detailed grasping style analysis was performed on trained rats in both the CST/RST injury group and the RST injury group. Although there were no changes in the grasping style of trained CST/RST injured rats, there were several changes found between trained and untrained RST injured rats. These changes were found in phases 4 and 6. Phase 4 is the grasping phase where the digits close around the pellet and phase 6 is the phase in which the palm is withdrawn from the opening of the chamber and turned so that the palm faces the rat's mouth. In trained RST injured rats, these phases were found to be either not present (score of 0) or abnormal (score of 0.5) when compared to trained rats. It is important to note that these alterations in the grasping style did not translate into enhancement (or detriment) in performing the task, as seen in the success rates between trained and untrained rats. Additionally, phase 3 of the grasp, which was nearly absent in all CST/RST injured rats, was present, though slightly abnormal (score below 1) in RST injured rats, showing that the arpeggio movement of the digits is possible with only an RST injury.

Cortical map changes are present following RST injury: In conjunction with an enhancement of functional recovery on the single pellet reaching task, we saw an increase in the occurrence of MEPs in trained CST/RST injured rats. Surprisingly, we also saw an increase in the occurrence of forelimb movements following RST injury following stimulation of the cortex contralateral to the SCI (i.e. projecting to the injured forelimb).

It is important to remember that the MEPs were elicited following cortical (CST) stimulation – a tract which was completely uninjured in these rats. Both RST trained and untrained rats had an increased occurrence of MEPs in comparison to control uninjured rats. Furthermore, RST trained rats had a reduced occurrence of MEPs in comparison to RST untrained rats. Again, we found no behavioural correlate for these changes in the single pellet grasping task, or in the horizontal ladder walking task.

The cortex ipsilateral to the SCI (i.e. projecting to the uninjured forelimb) also showed an increase in the occurrence of MEPs in the RST injury group. Both trained and untrained rats had an increase in the occurrence of MEPs in comparison to control uninjured rats. There was no further difference between trained and untrained rats seen in the ipsilateral cortical maps.

CST collaterals are increased following RST lesion: One mechanism which may explain the cortical map changes seen in the RST injury group is collateral sprouting. Again, it is important to remember that we counted the number of CST collaterals in these animals who only had an RST injury. We found an increase in the number of CST collaterals in RST untrained rats in comparison to control unlesioned rats. Surprisingly, we did not find this in RST trained rats. Although the sample size in the trained group is sufficient to detect statistical differences, and is in fact larger than the RST untrained group, it is likely the high variability which prevents finding statistical significance.

RST injury may have promoted the sprouting of CST fibers due to the availability of motor neurons once innervated by RST descending axons. Once the RST was lesioned, and the motor neurons they connected onto lost their input, they would be open for

reinnervation by descending tracts. The CST, a nearby tract, could have sprouted at any level (brain, brainstem, spinal cord rostral to the lesion, spinal cord caudal to the lesion) and we did indeed find an increased number of CST collaterals above the level of the SCI in these rats. This is one mechanism to explain the cortical map changes seen in RST injured rats.

3.4.3 CST injured rats

A small sample size in the CST trained group precluded statistical analyses of several key aspects in the study (most notably: cortical maps). Nevertheless, inclusion of the group demonstrates the importance of the CST in training-induced effects following SCI.

Single pellet reaching task success rate is not significantly increased following training: Although an increase in the success rate of CST injured rats was seen following training, there was not a significant increase in comparison to untrained rats. This is likely due to a low sample size (n=2) in the trained group, which precludes use of a non-parametric statistic, which should be used for a percentage score. Nevertheless, training returned CST injured animals to over 90% of their baseline scores, whereas non-trained CST injured rats only returned to less than 80% of their baseline scores.

CST trained rats perform significantly worse on the horizontal ladder walking task: Similar to the CST/RST trained rats, CST trained rats had a significant worsening of

performance on the horizontal ladder walking task in comparison to untrained CST injured rats, but did not show significant improvement on the grasping task.

Surprisingly, the number of errors seen in the CST injured rats was greater (non-significantly) than that of CST/RST animals. Although only errors of the preferred (injured) forelimb were counted, it is important to note that both of the CST injury groups (trained and untrained) had one rat with a bilateral CST lesion, which may have affected balance.

CST collaterals are non-significantly increased following CST injury: CST collaterals in both trained and untrained CST rats were increased compared to unlesioned rats. This did not reach statistical significance in either group, however, most likely due to a high variability in the groups. As explained previously, partial injuries to the CST have been shown to increase CST collaterals in the cervical enlargement (Bareyre et al., 2004; Fouad et al., 2001). Importantly, however, these studies subjected rats to an SCI at the thoracic level. In the current study, our rats were subjected to an SCI at the cervical enlargement. It may be that sprouting is reduced if the injury is nearby.

3.4.4 Contribution of the CST and RST in the single pellet reaching task

The contribution of both of these descending tracts on the performance of the single pellet reaching task has been well-described in a previous study (Whishaw et al., 1998). It is known that the CST and RST work in conjunction for a normal grasping pattern in rats. In animals with injuries to either tract alone, compensatory movement strategies are possible, yielding minimal alterations in performance of the task. In animals

with combined injuries to the CST and RST, skilled reaching success rates decline significantly, but the CST appears to have a stronger role in the execution of a reach. This may explain why training provided benefit in rats with an injury that included the CST. This may also suggest that the CST may be more responsive to reaching training, since its integrity has a higher impact in the performance of the task.

3.4.5 Concluding remarks

The current study is the first to report functional recovery following training of a volitional task controlled by descending input. We found this recovery only in CST/RST injured rats, and not in rats with injuries to either tract alone. Cortical map changes, and an increase in the number of CST collaterals are possible mechanisms for this recovery. Along with the recovery seen in CST/RST injured rats, we also observed a significant decline in the performance of another related task (the single pellet reaching task), leading us to hypothesize that a possible competition for neural substrates may underlie the behavioural results. Future studies may help verify this hypothesis.

3.5 Figures

Figure 3.1

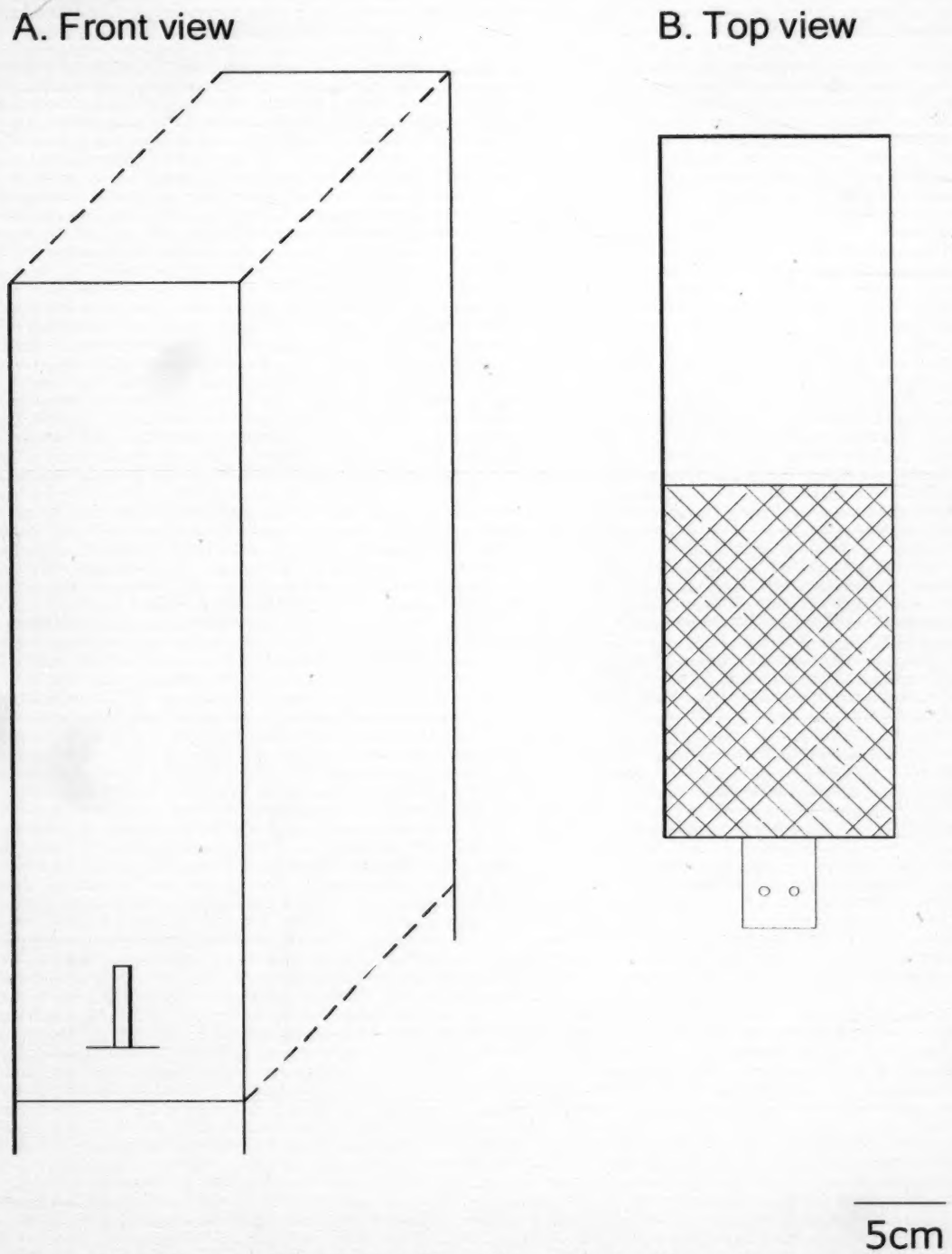


Figure 3.1. Schematic of the single pellet reaching task chamber used in the experiment.

Figure 3.2

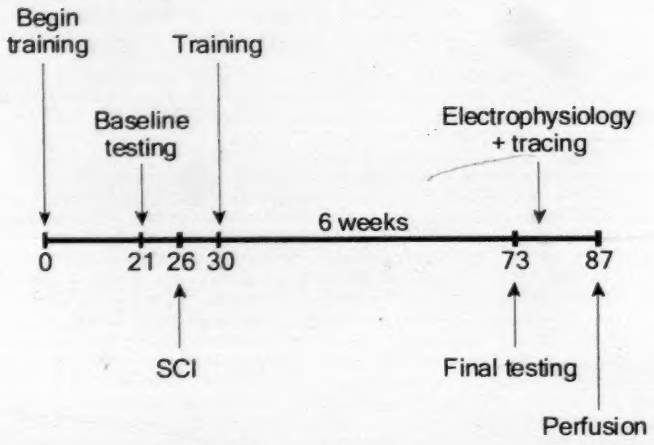


Figure 3.2. Timeline for the study. All animals were trained on the grasping task before being subjected to SCI. Following a 3 day recovery period, animals were separated into two groups (trained and untrained) and trained animals were trained on the grasping task for 6 more weeks. Final testing involved the single pellet grasping task (over 3 days) and the horizontal ladder walking task. Two weeks following electrophysiology and tracing, animals were perfused.

Figure 3.3

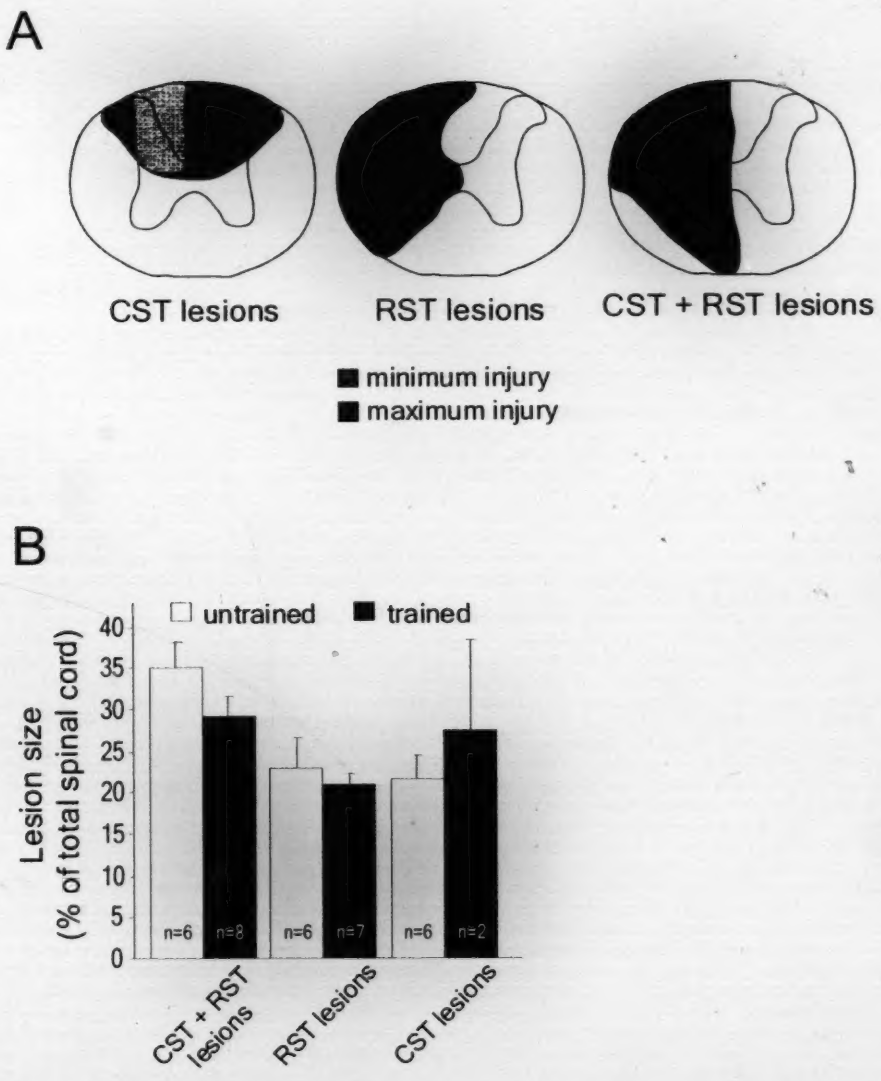


Figure 3.3. Lesion size was not changed by training. (A) Lesion size illustrations (minimum, average and maximum injury) in each group (trained and untrained animals pooled for each lesion group). (B) In each lesion group, lesions are not significantly different between trained and untrained rats. Data are presented as means ± standard error.

Figure 3.4

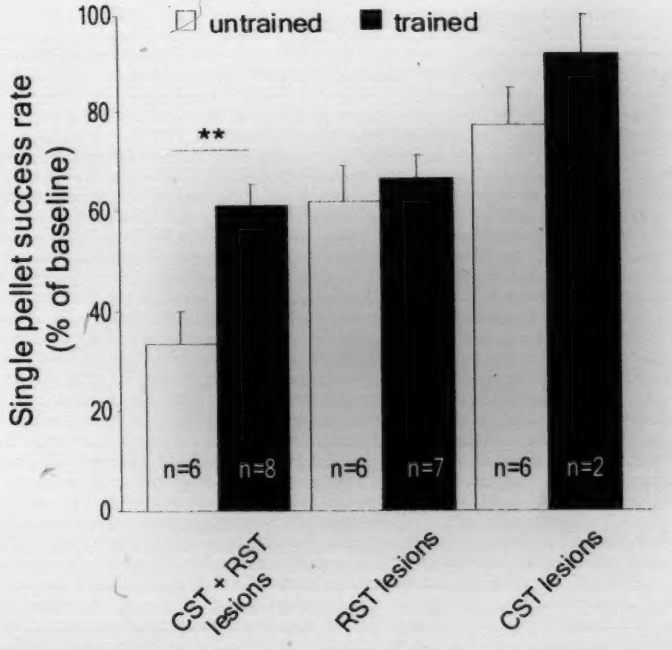


Figure 3.4. Success rate on the single pellet reaching task increases significantly following training in the CST/RST group. Following 6 weeks of training, animals in the CST/RST group improve significantly on the task compared to their untrained counterparts (p=.0047). The other two lesion groups show no statistically significant change following training on performance of this task. Error bars represent standard error of the mean. ** = $p<0.01$.

Figure 3.5

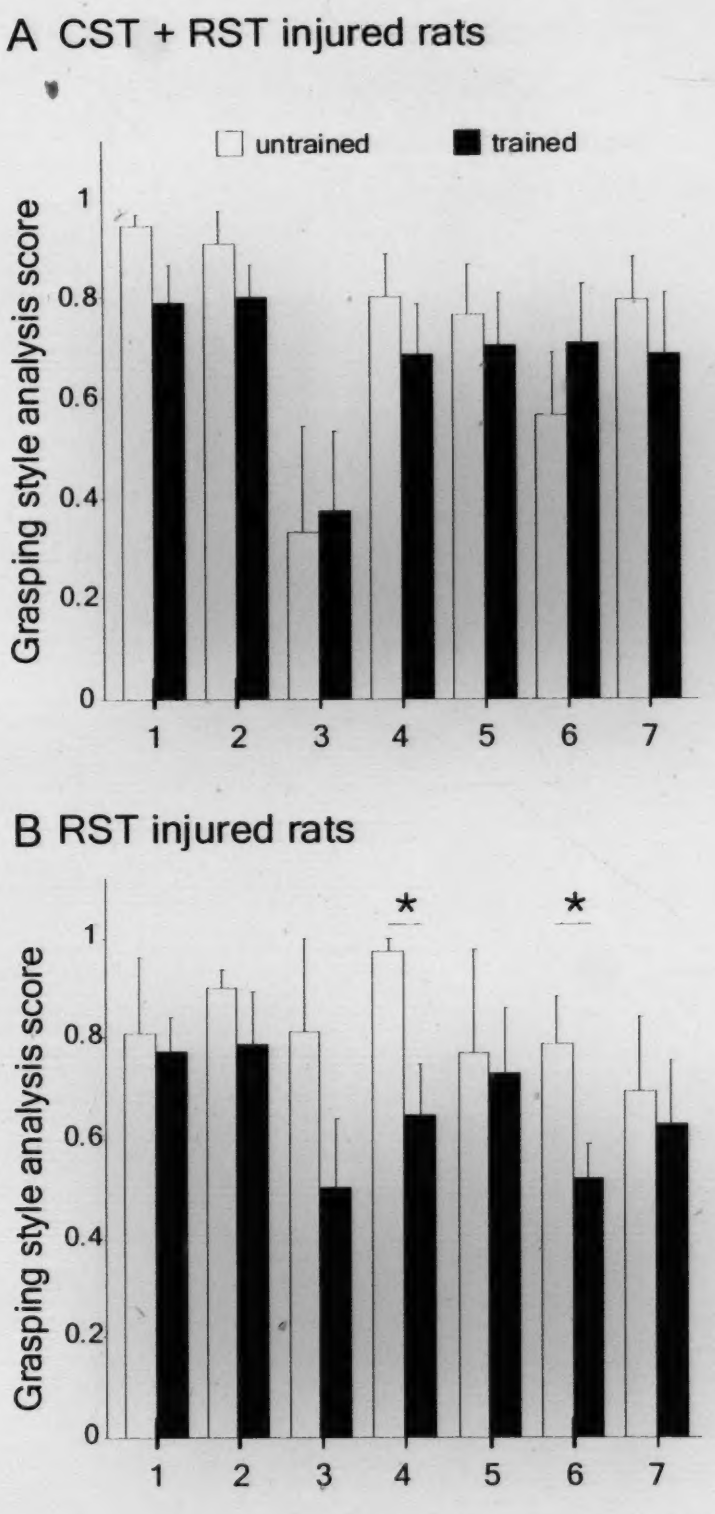

A CST + RST injured rats
untrained
trained
Grasping style analysis score
0
0.2
0.4
0.6
0.8
1
1
2
3
4
5
6
7
B RST injured rats
*
*
Grasping style analysis score
0
0.2
0.4
0.6
0.8
1
1
2
3
4
5
6
7

Figure 3.5. Detailed grasping style analysis shows significant changes in the grasping style of rats in the RST injury group following training. The 7 phases of the grasp analysed are detailed in *Methods*.(1) advance, (2) digits open, (3) pronation, (4) grasp, (5) supination I, (6) supination II, (7) release.(A) Animals in the CST/RST group did not change their grasping style at any phase of the grasp following training. As described in previous studies, lesions of both the CST and RST nearly completely abolishes pronation (phase 3) the phase in which the arpeggio movement of the digits can be seen in uninjured animals. (B) Animals in the RST group change their grasping style in two different phases but this did not translate into benefit or detriment in performing the task (as shown in Figure 3.3). Error bars represent standard error of the mean; * = $p<0.05$.

Figure 3.6

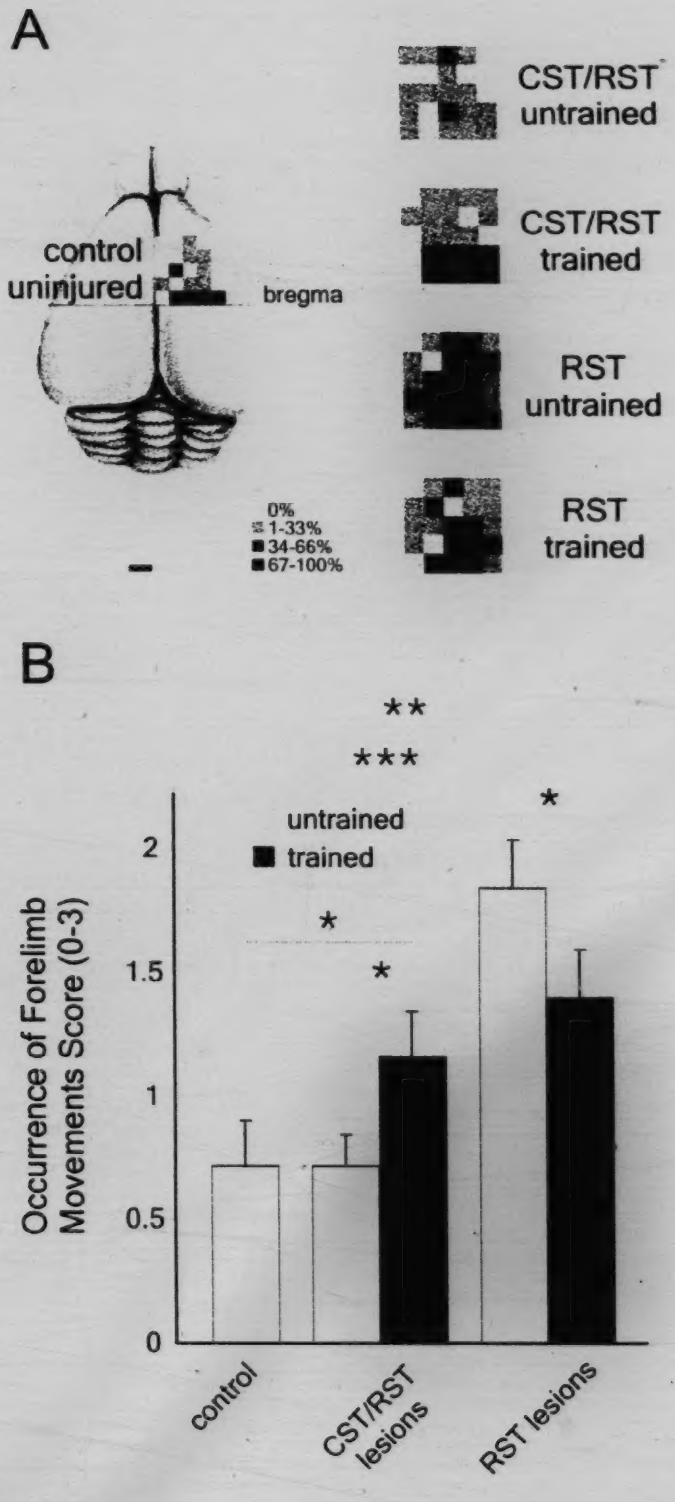

A
CST/RST untrained
CST/RST trained
RST untrained
RST trained
control uninjured
bregma
0%
1-33%
34-66%
67-100%
B
**
***
*
untrained
trained
*
*
Occurrence of Forelimb Movements Score (0-3)
2
1.5
1
0.5
0
control
CST/RST lesions
RST lesions

Figure 3.6. Cortical map following stimulation of the cortex contralateral to the injury. (A) The maps denote the occurrence ratio of forelimb movements per each stimulated spot (shown in the figure as a square) in the cortical forelimb area. Control map (obtained from uninjured rats) is shown in the middle. A 4-colour colour code was used in the maps denoting high (red) and low (yellows) occurrence ratios of forelimb movements at each spot. To quantify differences in the maps, the colour codes were transferred into numbers (0-3) and a matched pairs t-test showed significant differences between most injured subgroups to control animals (B). CST/RST trained rats ($p=0.0295$), RST untrained rats ($p<0.0001$) and RST trained rats ($p=0.0025$) all had significantly higher occurrence ratios of forelimb movements compared to uninjured control rats. Additionally, CST/RST trained rats ($p=0.0413$) and RST untrained rats ($p=0.0479$) had significantly higher occurrence ratios in comparison to their lesioned counterparts. Black bar denotes the side of the spinal cord injury. Error bars represent standard error of the mean; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

Figure 3.7

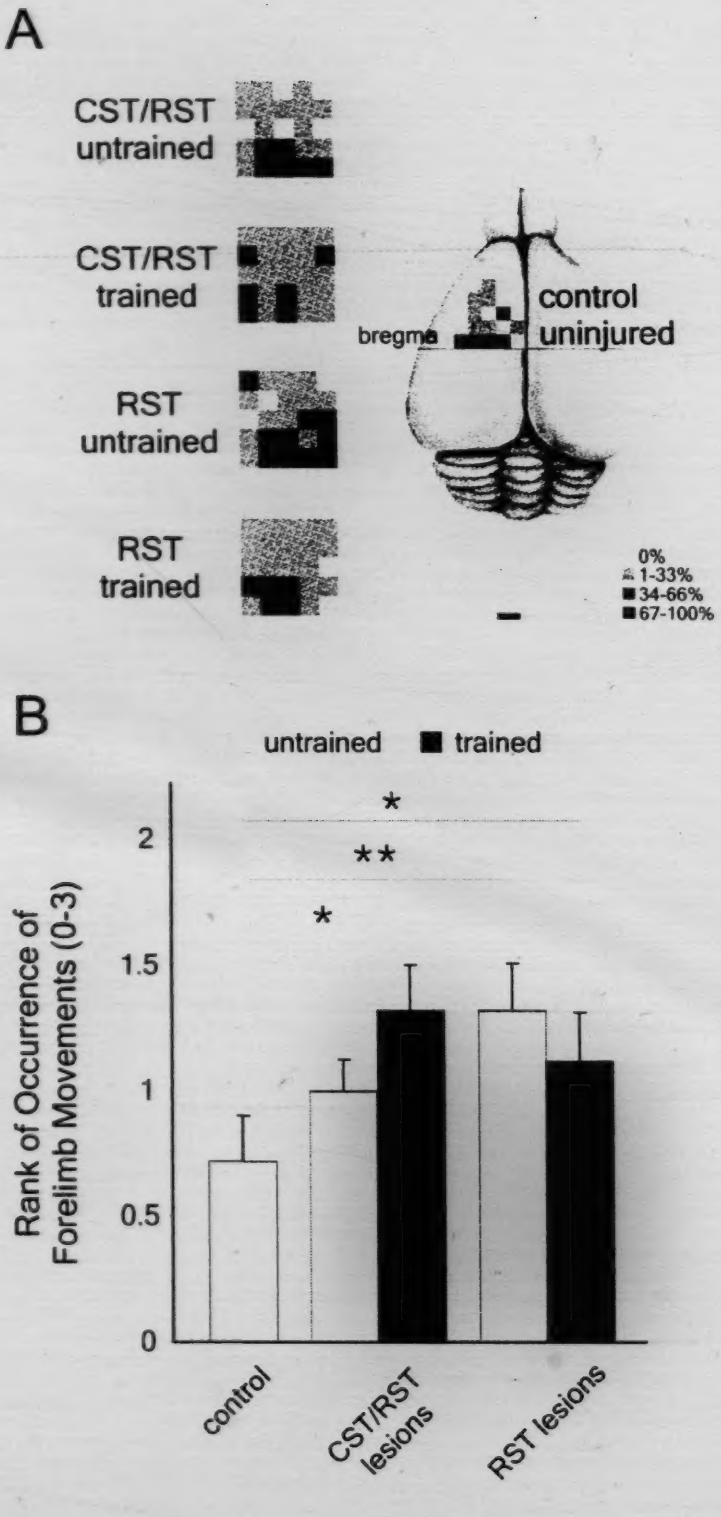

A
CST/RST untrained
CST/RST trained
RST untrained
RST trained
bregma
control uninjured
0%
1-33%
34-66%
67-100%
B
untrained
trained
Rank of Occurrence of Forelimb Movements (0-3)
2
1.5
1
0.5
0
*
**
*
control
CST/RST lesions
RST lesions

Figure 3.7. Cortical map changes following stimulation of the cortex ipsilateral to the injury. (A) The maps denote the occurrence ratio of forelimb movements per each spot in the cortical forelimb area. (B). CST/RST trained ($p=0.0134$), RST untrained ($p=0.0067$) and RST trained ($p=0.0448$) rats all had an increase in the occurrence of forelimb movements compared to uninjured control rats. No differences existed between trained and untrained rats in either of the lesion paradigms. Black bar denotes the side of the spinal cord injury. Error bars represent standard error of the mean; * = $p<0.05$, ** = $p<0.01$.

Figure 3.8

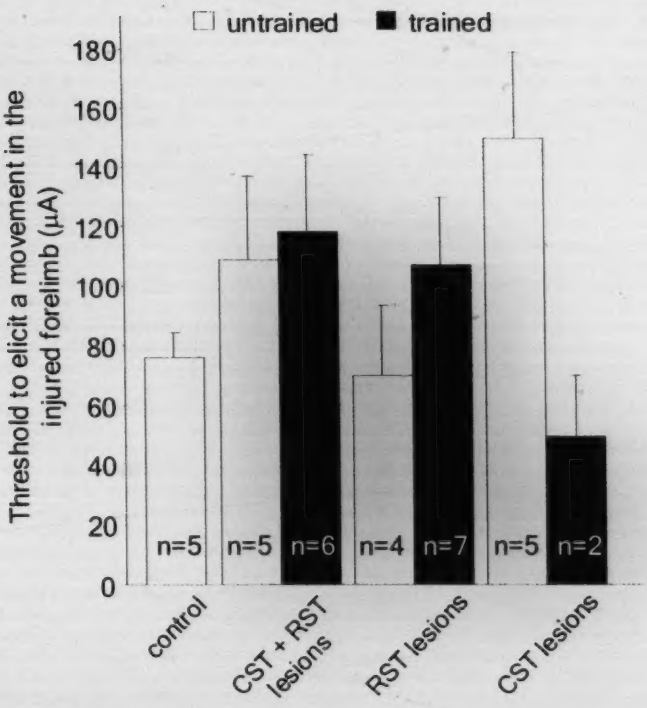
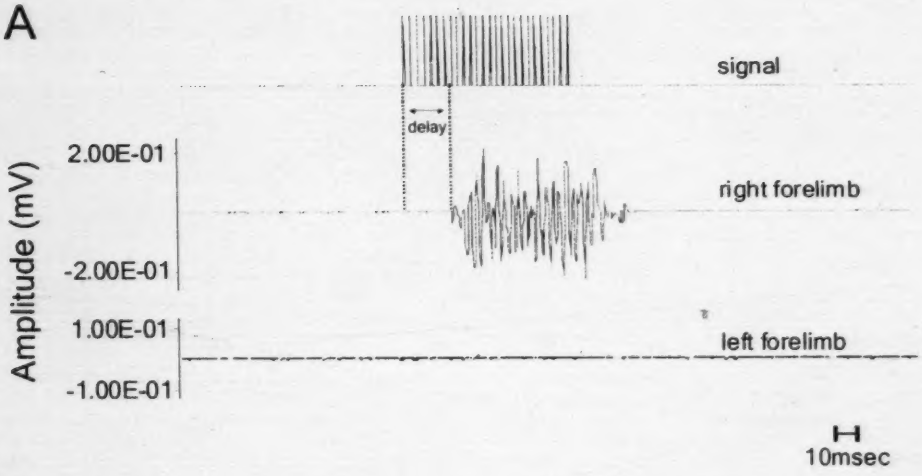


Figure 3.8. Minimum threshold to elicit a movement of the injured forelimb does not differ significantly following training in any of the injury subgroups. There were no significant changes in minimum threshold to elicit forelimb movements in any injury group following training or in any treatment/injury group compared to control rats. Error bars represent standard error of the mean; * = $p<0.05$.

Figure 3.9

A



B

	cortex i	wrist c	cortex c	wrist i	cortex c	wrist c	cortex i	wrist i
CST inj. untrained	20.0		15.4		-		24.2	
CST inj. trained	18.6		19.8		28.5		30.8	
RST inj. untrained	14.7		11.0		-		-	
RST inj. trained	18.5		11.7		43.2		-	

Figure 3.9. Delays to elicit motor evoked potentials are not changed with training. A) Representative trace obtained from rats under isofluorane anesthesia. B) Data presented from CST/RST (combined with CST only) and RST only trained and untrained rats. i=ipsilateral to injury, c=contralateral to injury, cortex=side of the cortex stimulated, w=forelimb which was activated (ipsilateral or contralateral to injury). -=no data fitting criteria.

Figure 3.10

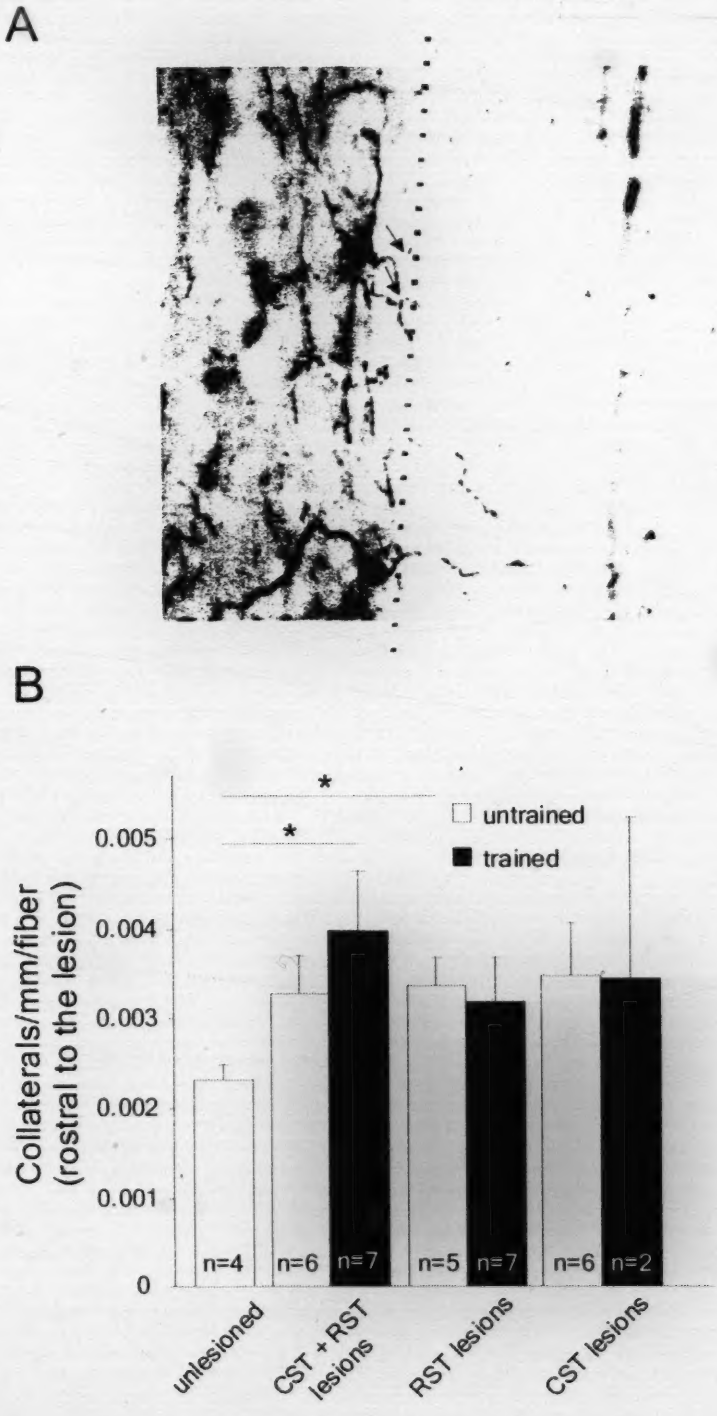

A
B
Collaterals/mm/fiber
(rostral to the lesion)
0.005
0.004
0.003
0.002
0.001
0
*
*
untrained
trained
n=4
n=6
n=7
n=5
n=7
n=6
n=2
unlesioned
CST + RST
lesions
RST lesions
CST lesions

Figure 3.10. The number of CST collaterals found rostral to the injury is increased with training (compared to uninjured rats) in the CST/RST injury group. A) Horizontal section of the cervical spinal cord showing collaterals emerging from the main CST into the grey matter. Arrows point to individual collateral sprouts. B) The number of collaterals emerging from the injured CST is increased significantly from uninjured (control) rats following training ($p=0.048$) but is not significantly increased in the no training group ($p=0.10$) in the CST/RST injured group. Number of CST collaterals found rostral to the injury. Furthermore, CST collateral counts increased following RST injury. Collateral counts did not differ between trained and untrained animals in any of the subgroups. Error bars represent standard error of the mean; * = $p<0.05$.

Figure 3.11

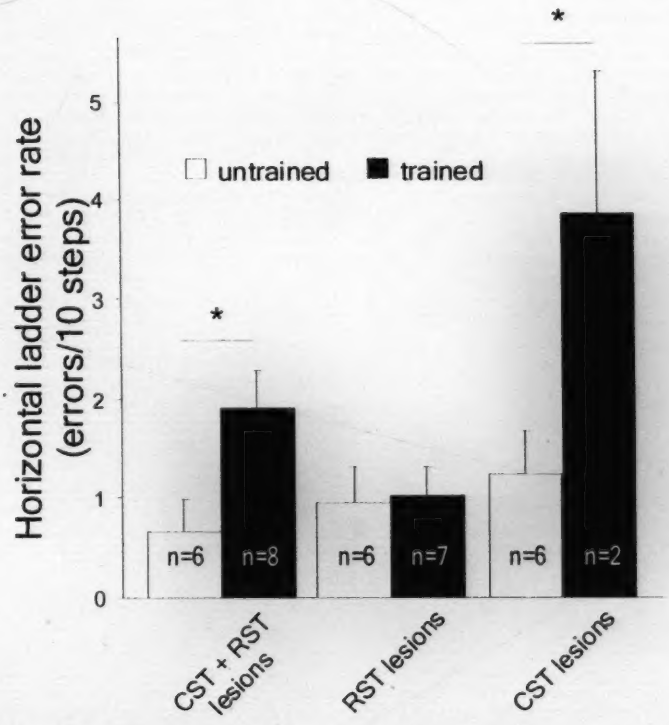


Figure 3.11. Forelimb slips and falls (errors) increase significantly in the CST/RST and CST only injury groups following grasp training. Trained animals in both the CST/RST ($p=0.033$) and CST ($p=0.048$) injury groups showed a significant decline in performance of the horizontal ladder walking task compared to their untrained counterparts. Rats in the RST only group do not perform significantly differently on the task following training. Error bars represent standard error of the mean; * = $p<0.05$.

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CHAPTER 4

DISCUSSION

DISCUSSION

In the previous chapters, two separate studies (see Figure 4.1 for a schematic of the first project and Figure 4.2 for a schematic of the second project) attempting to promote functional recovery following SCI have been described. The interventions in both of the studies promoted plasticity following SCI in the form of cortical map changes and/or changes in the number of collaterals emerging from the CST.

4.1 MAJOR FINDINGS

The first study described above expanded on previous work describing the spontaneous formation of new intraspinal circuits following SCI (Fouad et al., 2001, Bareyre et al., 2004). In Bareyre (2004), the number of branches emanating from the main CST tract into the grey matter and the number of contacts from these branches onto propriospinal interneurons spontaneously increased following SCI when compared to uninjured animals. In the first study detailed above (Chapter 2), it was found that BDNF treatment to the cell body of an injured descending tract (the CST) can increase the number of collaterals (branches) from that tract into the grey matter of the cervical spinal cord. Contrary to the original work, the injury size in the current study was much larger and most likely destroyed more interneurons, making it less likely for collaterals to find a target, possibly causing them to abort their growth.

BDNF treatment was also found to increase the number of contacts (from these increased collaterals) onto propriospinal interneurons in the cervical spinal cord. As with the collaterals, injury alone was not sufficient to significantly increase the number of

contacts compared to uninjured rats, but this is also likely due to a reduction in the number of interneurons available for connections.

Importantly, however, animals with more corticospinal/propriospinal interneuron contacts, improved behaviourally compared to animals with fewer contacts (as seen with a statistically significant correlation between number of contacts and number of errors on the horizontal ladder walking task.) This study showed that a treatment administered to the cell body of a descending tract could increase targeted sprouting and promote meaningful connections within the injured spinal cord.

NT-3 application to the cervical enlargement had no discernable effect on the growth of collaterals or on their connections onto interneurons. Previous studies have reported a significant increase in the number of collaterals following NT-3 application, but these studies have several methodological differences to the current study. In one of the previous reports (Schnell et al., 1994) NT-3 was applied directly at the lesion site. In another study (Zhou et al., 2003), NT-3 levels were increased by use of a vector applied into the hindlimb musculature of rats, which would increase NT-3 levels in the cell bodies of motor neurons at a location quite caudal to the SCI. It appears that NT-3 application following SCI will provide benefit (i.e. increase collaterals) if it is given at the lesion site or if it is expressed in motor neuron cell bodies (which would act as a signal for axons to innervate the motor neuron, and would draw it nearer). A recent publication (Hagg et al., 2005) has reported a significant decrease in the number of collaterals following SCI and NT-3 application to the cervical enlargement. Although we also show a decline in the number of CST collaterals in NT-3 treated rats with SCI, our

results did not reach statistical significance. This lack of statistical significance was likely due to a small sample size in the control group (n=3).

In the second study detailed above, specific (grasp) training was administered to animals following partial SCI with the goal to promote functional recovery. We tested three lesion groups: combined CST/RST injury, CST only injury and RST only injury. Recovery in the single pellet grasping task was found following training, but only in animals in the CST/RST group. In those same animals, we surprisingly also found a significant decrease in the performance of a related task, the horizontal ladder walking task, with their trained forelimb. Trained rats in the CST only group also showed a significant increase in the number of errors made in crossing the horizontal ladder compared to untrained CST injured rats.

The two mechanisms for recovery in the CST/RST trained group appear to be changes in the forelimb area of the cortex and sprouting of the CST. Specifically, trained animals in the CST/RST group had an increase in the occurrence of forelimb movements of their trained/injured forelimb following cortical stimulation in comparison to both uninjured rats and injured (CST/RST) untrained rats. Although it did not lead to a change in the performance of the single pellet reaching task or the horizontal ladder walking task, RST injured rats also had an increase in cortical maps compared to uninjured rats.

In addition to cortical map changes, there was also sprouting of the injured CST in CST/RST trained rats, and RST untrained rats in comparison to uninjured control rats. Although there was an increase in the number of CST collaterals in CST/RST untrained rats compared to uninjured rats, this effect did not reach statistical significance. Also in line with the cortical map increases seen in RST injured rats, RST untrained rats also had

an increased number of collaterals emanating from the CST on the injured side of the spinal cord (compared to uninjured rats). Furthermore, although we did not perform any further histological analyses, sprouting within the cortex may have occurred, leading to an increase in the occurrence of forelimb movements following cortical stimulation. Additionally, although we found sprouting of the CST within the cervical spinal cord in CST/RST trained rats, there may have also been sprouting at the level of the brainstem.

The results raise the idea of a limited capacity for connections within the brain and the possibility that there is a competition for these neural substrates. Since training of one task resulted in improvements in that task at the cost of a decreased performance in another task, this appears to be the most likely scenario. Unfortunately, very little research exists on this topic, so it is impossible to compare our results to previous results. Accordingly, much further research is needed to confirm that this is in fact the case.

4.2 Applications of the Results

The results detailed above are novel findings showing that plasticity is possible following injury in the adult central nervous system. Importantly, both studies can possibly be applied to the human condition (i.e. applied as a treatment following SCI).

Although global application of a pharmacological treatment (i.e. I.V. infusion) may be a fast and easy approach to get a drug into the system, but it will also yield side effects very frequently. To extrapolate the data from the first study into the human condition, if BDNF were given globally to patients, it may be assumed that it would globally increase sprouting of fibers. An important thing to remember is that not all tracts within the spinal cord (and brain, for that matter) function towards motor control.

Sprouting of sensory fibers, for example, may lead to an increased sensation of pain, a very serious side effect which is often seen spontaneously following SCI. Another serious clinical problem arising from sprouting of sensory fibers is autonomic dysreflexia which, again, is sometimes found in patients following SCI. Autonomic dysreflexia is an overactivity of the autonomic nervous system and is manifested most commonly in an inability to regulate blood pressure leading quite often to strokes.

Application of a pharmacological agent specifically to a single tract or several distinct tracts may, in theory, bypass this problem. Clearly, it is not possible to deliver an agent to the brain of a human subject with SCI using a mini-pump implanted into the brain (due to trauma to the brain and also risk of infection), but the results above show that in theory specific application of neurotrophic factors is possible.

The second project also yields results applicable to human SCI. Following injuries affecting the forelimb (e.g. SCI and stroke) patients are often subjected to rehabilitation training involving CIMT with or without grasp training or repetitive training of other forelimb movements. These studies will often not access the applicability of training of these movements onto activities of daily living or even on other related tasks. Our study is the first to show detriment on an untrained (but related) task along with the benefit of a trained task. It is important to remember that the results presented here do not involve changes in the properties of a pattern generating network (as with the work on spinalized cats trained either to walk or stand) but directly on two descending tracts.

4.3 Future Directions

The results from both Chapter 2 and Chapter 3 warrant further examinations in new projects. In the next section, I will detail the future studies which should be conducted based on the results from each of the chapters.

4.3.1 Chapter 2 Future Directions

In this study, we reported a significant increase in the number of CST collaterals emerging rostral to a thoracic over-hemisection lesion, but only in BDNF-treated rats. NT-3 treated rats had a non-significant reduction in the number of CST collaterals emerging into the cervical enlargement. Hagg and colleagues (2005) have shown that NT-3 can actually cause a *significant* decline in the number of CST collaterals. One of the first future studies that should be completed is to add more control animals to the study, as there was only an *n* of 3 in this group. Additionally, the location of the application of NT-3 should also be tested, as the extent of collateral sprouting varies depending on the location of application (as stated in section 4.1).

In addition, this same experiment should be conducted in rats with a complete spinal cord transection. In a previous study, Bregman and colleagues (Coumans et al., 2001) showed that following a complete transection and application of a CNS transplant (into the cavity), propriospinal axons were able to regrow through the transplant. Following neurotrophins application in these same animals, descending supraspinal axons were also found to regrow through the graft. It would, therefore, be interesting to see whether neurotrophins could then enhance the formation of intraspinal circuits following complete SCI.

4.3.2. Chapter 3 Future Directions

CST/RST Group: In Chapter 3, we showed that grasping training could provide significant functional recovery to rats, but in those rats with a combined CST/RST injury. Several future studies should be completed to expand upon the findings of the current report. First of all, to test the hypothesis of competition for neural substrates, injured rats should be trained on the single pellet reaching task and also tested on a task requiring input from a separate tract. For example, overground walking relies primarily on reticulospinal tract (RtST) input. If there is in fact competition for neural substrates between two related tasks (grasping and ladder walking) following training of one of the tasks, walking performance should not be altered.

Another way to test the theory of the competition for neural substrates is to train injured rats on both the single pellet reaching task and the horizontal ladder walking task. Several outcomes are possible. If there is a competition for neural substrates, it could be assumed that trained rats would perform better than untrained rats on both of the tasks, but at a level lower than rats trained on either task alone.

Furthermore, it is important that the delay between SCI and training be examined. It is well established that there is a sensitive period following cortical injury where therapeutic interventions involving increased activity of the injured limb (e.g. CIMT) may actually be deleterious. This has not been fully examined following SCI. Although we found no evidence that training at 3 days following injury provided a detriment, delaying the training past a possible post-lesion sensitive period may provide even more benefit.

RST Group: There are several future studies which can be completed using this injury paradigm to help fully understand the implication of the RST in functional recovery following SCI. Most importantly, the number of RST collaterals emanating from the injured tract needs to be determined. In the current study, we found increases in CST collaterals (in comparison to control uninjured rats) in the CST/RST injury group following training. The number of CST collaterals we found in this group is higher (non-significantly) than those found in RST injured animals. It may be that injury of a tract is required to promote the highest level of sprouting in a tract. If that were the case, then the RST may have sprouted above or below the lesion.

Another study which may be completed is looking at the red nucleus itself following injury and training. It may be that the red nucleus has decreased atrophy, or increased cell size following training in comparison to untrained rats. Furthermore, thresholds, motor maps, and delays to evoke an MEP elicited from red nucleus stimulation should also be completed.

CST Group: Clearly, a larger sample size in the CST trained group is needed to make conclusions on the impact of training on an injury of the CST. Currently, a low sample size did not allow for the assembly of cortical maps and may have prevented finding a statistically significant improvement in performance of the single pellet grasping task in trained rats.

All Groups: In the previous sections, I have listed future directions specific to each lesion paradigm. Further studies (with any/all of the lesion paradigms) should also be

completed. Firstly, a study which has already started is attempting to determine which factors (neurotrophins, growth associated proteins, etc) are upregulated following grasping training. The majority of studies reporting an increase in neurotrophins and growth associated proteins following physical activity use tissue from rats that have undergone treadmill training or running wheel training. To date, no study has addressed the effects of increased forelimb activity on neurotrophins or growth associated proteins.

Secondly, the effects of training plus various pharmacological agents should be assessed. It may be the case that although training alone does have beneficial effects, more powerful interventions are needed to combat the inhibitory environment of the damaged CNS. It is likely that a combinatorial treatment regimen would prove to be most successful in treating SCI since it is such a multifaceted problem. To do this, agents which neutralize myelin based protein inhibitors, neurotrophic factors, chondroitinase and/or inhibitors of the Rho pathway (see Chapter 1) may be administered at the same time as training.

4.4 Concluding Remarks

There are several main findings presented in the previous chapters. Firstly, BDNF treatment to the cell body of injured CST axons can promote the sprouting of these axons into the grey matter and the connections of these axons onto descending interneurons. The increased connections found after treatment correlated well with performance on a behavioural task.

Second, specific training of a task administered following cervical SCI can improve performance of that task. This improvement, however, may come at the cost of a

decreased performance on other closely related tasks. Following CST/RST injury and training, the forelimb cortical area of rats is able to change (possibly due to increases in the number of CST collaterals) yielding an increased occurrence of forelimb movements in the injured forelimb.

4.5 FIGURES

Figure 4.1

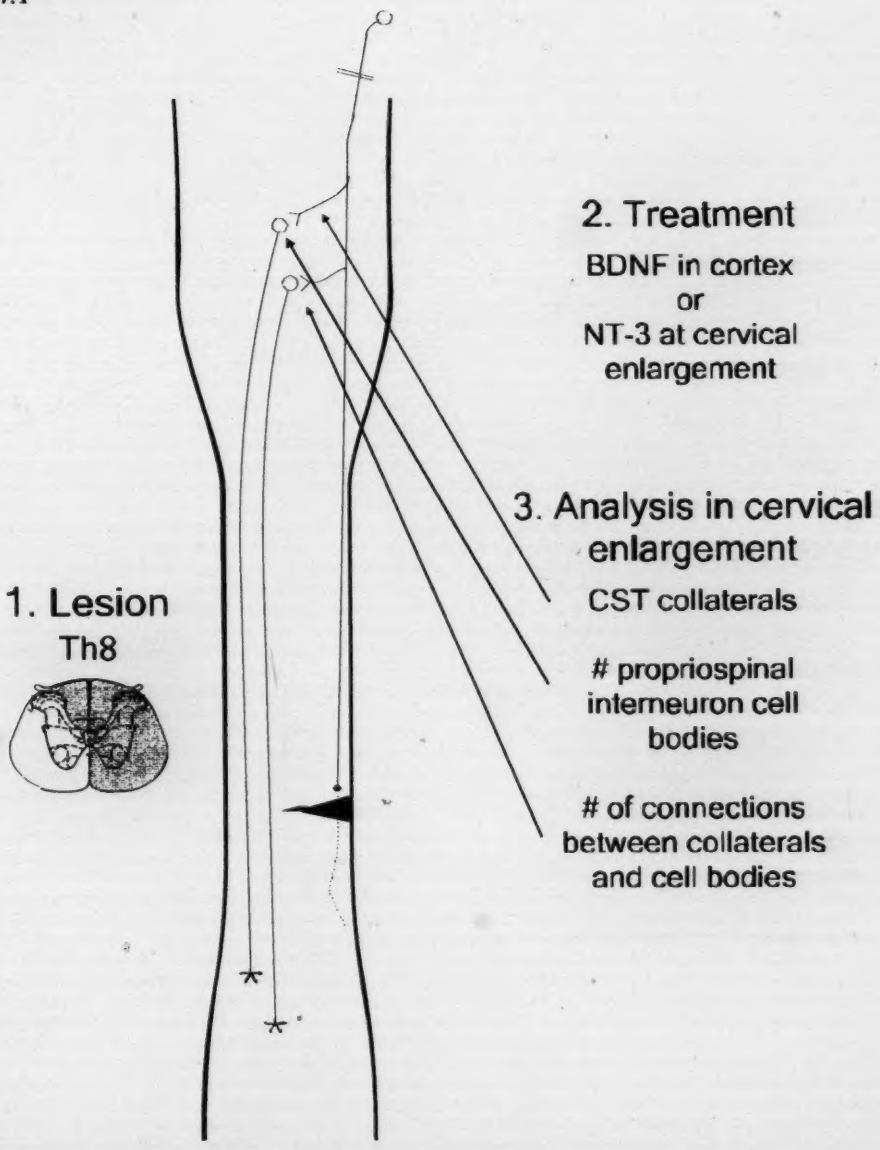

Figure 4.1. Schematic of the study from Chapter 2. Neurotrophins were used to assess their effects on both sprouting of the CST and the number of connections found between these sprouts and long descending propriospinal interneurons in the cervical enlargement, following a partial mid-thoracic injury.

Figure 4.2

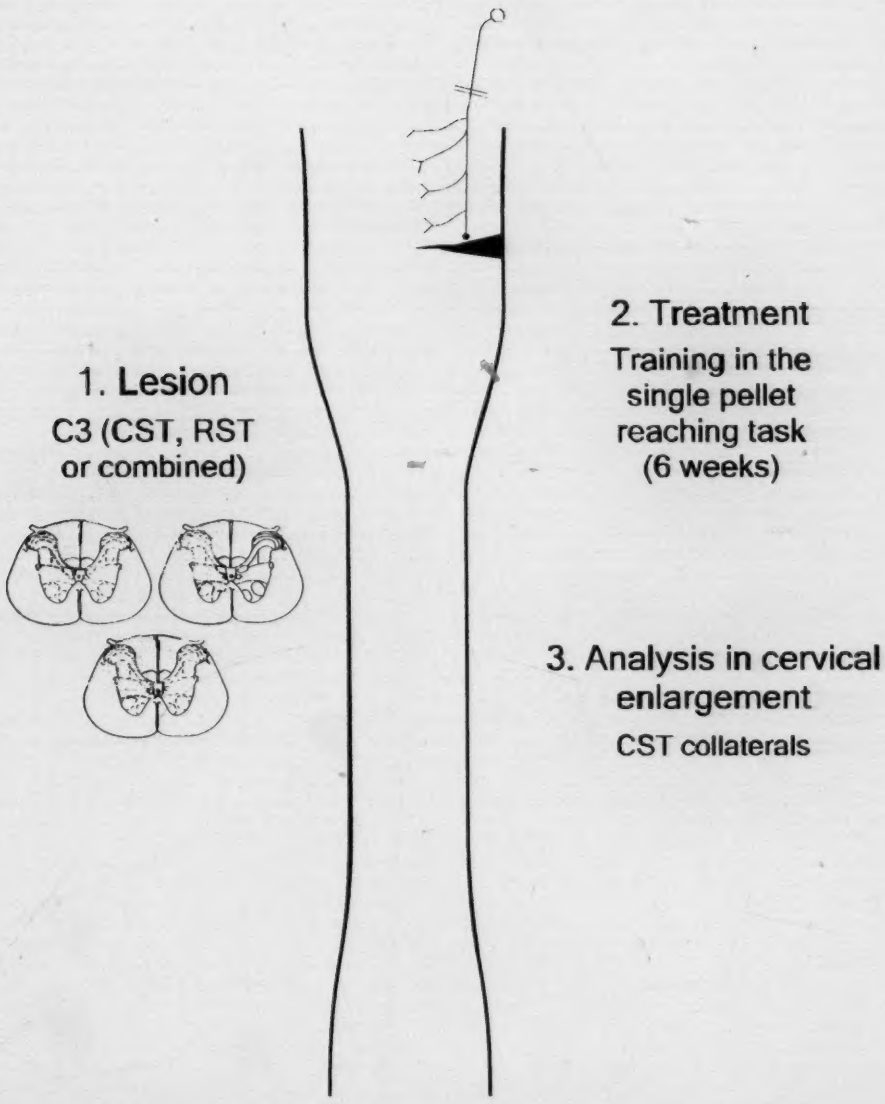
1. Lesion
C3 (CST, RST or combined)
2. Treatment
Training in the single pellet reaching task (6 weeks)
3. Analysis in cervical enlargement
CST collaterals

Figure 4.2. Schematic of the study from Chapter 3. Exercise was used as a treatment following partial SCI at the cervical level. The number of CST collateral sprouts were assessed in the cervical enlargement.

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